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(57) Abstract

The invention is directed to helper adenoviruses which facilitate the production of pseudoadenoviral vectors (PAV) but which cannot be packaged into viral particles. The invention is further directed to novel PAV producer cell lines expressing DNA binding and/or repressor proteins that prevent the packaging of the helper viruses. The invention is also directed to methods for the production of PAV in such cell lines with minimal contamination from helper viruses.

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Methods for Pseudoadenoviral Vector Production

Introduction

The invention is directed to novel helper adenoviruses which facilitate the production of pseudoadenoviral vectors (PAV) but which cannot be packaged into viral particles. The invention is further directed to novel PAV producer cell lines expressing DNA binding and/or repressor proteins that prevent the packaging of the helper viruses. The invention is also directed to methods for the production of PAV in such cell lines with minimal contamination from helper viruses.

Background of the Invention

Pseudoadenoviral vectors (PAV) are adenoviral vectors derived from the genome of an adenovirus which contain the minimal cis-acting nucleotide sequences required for the replication and packaging of the vector genome and which can contain one or more transgenes (see, e.g., allowed U.S. Application Serial No. 08/895,194) incorporated herein by reference). Such PAVs are advantageous because the transgene carrying capacity of the vector is optimized (up to 36Kb in size), while the potential for host immune reaction to viral proteins or for the generation of replication-competent viruses is reduced. PAVs contain the adenoviral 5' and 3' inverted terminal repeat (ITR) nucleotide sequences containing origins of replication, the 5' cis-acting packaging signal of the viral genome, and can accomodate one or more transgenes with operably linked expression elements. These minimal viral nucleotide sequences retained in PAVs are required in cis for the replication and packaging of the PAV genome into viral particles. In addition, the production of PAVs requires the provision of a helper adenovirus to supply the viral proteins required for replication of the PAV genome and assembly of the viral particles.

PAV vectors have been designed to take advantage of the desirable features of adenovirus which render it a suitable vehicle for gene transfer, as evidenced by studies with first and second generation adenoviral vectors. Adenovirus is a non-enveloped,

nuclear DNA virus with a genome of about 36 kb, which has been well-characterized through studies in classical genetics and molecular biology (Horwitz, M.S., "Adenoviruses," in Virology, 3rd edition, Fields et al., eds., Raven Press, New York, 1996; Hitt, M.M. et al., "Adenovirus Vectors," in The Development of Human Gene Therapy, Friedman, T. ed., Cold Spring Harbor Laboratory Press, New York, 1999). The viral genes are classified into early (known as E1-E4) and late (known as L1-L5) transcriptional units, referring to the generation of two temporal classes of viral proteins. The demarcation between these events is viral DNA replication. The human adenoviruses are classified into numerous serotypes (approximately 47, numbered accordingly and organized into 6 subgroups: A, B, C, D, E and F), based upon properties including hemaglutination of red blood cells, oncogenicity, DNA base and protein amino acid compositions and homologies, and antigenic relationships.

Recombinant adenoviruses have several advantages for use as gene transfer vectors, including tropism for both dividing and non-dividing cells, minimal pathogenic potential, ability to replicate to high titer for preparation of vector stocks, and the potential to carry large inserts (Hitt, et al. supra); Berkner, K.L., Curr. Top. Micro. Immunol. 158:39-66, 1992; Jolly, D., Cancer Gene Therapy 1:51-64, 1994).

The cloning capacity of an adenovirus vector to date has been proportional to the size of the adenovirus genome present in the vector. For example, a cloning capacity of about 8 kb results from the deletion of regions of the virus genome which are dispensable for virus growth, e.g., E3, and the deletion of a genomic region such as E1 whose function may be restored in trans from a complementing cell line such as 293 cells (Graham, F.L., J. Gen. Virol. 36:59-72, 1977). Such E1-deleted vectors are rendered replication-defective, a desirable attribute for a gene transfer vector. The upper limit of vector DNA capacity for optimal carrying capacity is about 105%-108% of the length of the wild-type genome. Further adenovirus genomic modifications are possible in vector design using cell lines which supply other viral gene products in trans, e.g., complementation of E2a (Zhou et al., J. Virol. 70:7030-7038, 1996), complementation of E4 (Krougliak et al., Hum. Gene Ther. 6:1575-1586, 1995; Wang et al., Gene Ther. 2:775-783, 1995), or

complementation of protein IX (Caravokyri et al., J. Virol. 69:6627-6633, 1995; Krougliak et al., Hum. Gene Ther. 6:1575-1586, 1995).

However, maximal carrying capacity can be achieved with the use of adenoviral vectors containing deletions of most viral coding sequences, including PAVs (allowed U.S. Patent Application Serial No. 08/895,194; Kochanek et al., Proc. Natl. Acad. Sci. USA 93:5731-5736, 1996; Parks et al., Proc.Natl.Acad.Sci. USA 93:13565-13570, 1996; Lieber et al., J.Virol. 70:8944-8960, 1996; Fisher et al., Virology 217:11-22, 1996; U.S. Patent No. 5,670,488; PCT Publication No. WO96/33280, published October 24, 1996; PCT Publication No. WO96/40955, published December 19, 1996; PCT Publication No. WO97/25446, published July 19, 1997; PCT Publication No. WO95/29993, published November 9, 1995; PCT Publication No. WO96/13597, published May 9, 1996; PCT Publication No. WO97/00326, published January 3, 1997; Morral et al., Hum.Gene Ther. 10:2709-2716, 1998; Burcin et al., Proc. Natl. Acad. Sci. USA 95:355-360, 1999).

A wide variety of transgenes (foreign nucleic acids) have been delivered to various target cells by first and second-generation adenoviral vectors, illustrating the heterogeneity of adenoviral vector transduction. Such transgenes include p53 (Wills et al., Human Gene Therapy 5:1079-188, 1994); dystrophin (Vincent et al., Nature Genetics 5:130-134, 1993; erythropoietin (Descamps et al., Human Gene Therapy 5:979-985, 1994; ornithine transcarbamylase (Stratford-Perricaudet et al., Human Gene Therapy 1:241-256, 1990; We et al., J. Biol. Chem. 271;3639-3646, 1996;); adenosine deaminase (Mitani et al., Human Gene Therapy 5:941-948, 1994); interleukin-2 (Haddada et al., Human Gene Therapy 4:703-711, 1993); and α1-antitrypsin (Jaffe et al., Nature Genetics 1:372-378, 1992); thrombopoietin (Ohwada et al., Blood 88:778-784, 1996); and cytosine deaminase (Ohwada et al., Hum. Gene Ther. 7:1567-1576, 1996).

The particular tropism of adenoviruses for cells of the respiratory tract has relevance to the use of adenoviral vectors for gene transfer in cystic fibrosis (CF), which is the most common autosomal recessive disease in Caucasians. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that disturb the cAMP-regulated Cl^- channel in airway epithelia result in pulmonary dysfunction (Zabner et al., Nature Genetics 6:75-83, 1994). Adenovirus vectors engineered to carry the CFTR

gene have been developed (Rich et al., Human Gene Therapy 4:461-476, 1993) and studies have shown the ability of these vectors to deliver CFTR to nasal epithelia of CF patients (Zabner et al., Cell 75:207-216, 1993), the airway epithelia of cotton rats and primates (Zabner et al., Nature Genetics 6:75-83, 1994), and the respiratory epithelium of CF patients (Crystal et al., Nature Genetics 8:42-51, 1994). Recent studies have shown that administering an adenoviral vector containing a DNA sequence encoding CFTR to airway epithelial cells of CF patients can restore a functioning chloride ion channel in the treated epithelial cells (Zabner et al., J. Clin. Invest. 97:1504-1511, 1996; U.S. Patent No. 5,670,488, issued September 23, 1997). Persistent expression of CFTR from adenoviral vectors which results in the establishment of functional chloride channels in the airway epithelium of immunocompetent animals has recently been achieved (Scaria et al., J.Virol. 72:7302-7309, 1998).

The use of first and second generation adenovirus vectors in gene transfer studies to date indicates that persistence of transgene expression in target cells and tissues is often transient. This is at least partly due to the generation of a cellular immune response to viral proteins which are expressed even from a replication-defective vector, triggering a pathological inflammatory response which may destroy or adversely affect the adenovirus-infected cells (Yang et al., J. Virol. 69:2004-2015, 1995; Yang et al., Proc. Natl. Acad. Sci. USA 91:4407-4411, 1994; Zsengeller et al., Hum Gene Ther. 6:457-467, 1995; Worgall et al., Hum. Gene Ther. 8:37-44, 1997; Kaplan et al., Hum. Gene Ther. 8:45-56, 1997). Because adenovirus does not integrate into the cell genome, an adverse immune response poses a serious obstacle for high dose administration of an adenovirus vector or for repeated administration (Crystal, R., Science 270:404-410, 1995).

In order to circumvent the host immune response which limits the persistence of transgene expression, various strategies have been employed, generally involving either the modulation of the immune response itself or the engineering of a vector that decreases the immune response. The administration of immunosuppressive agents together with vector administration has been shown to prolong transgene persistence (Fang et al., Hum. Gene Ther. 6:1039-1044, 1995; Kay et al., Nature Genetics 11:191-197, 1995; Zsellenger et al., Hum. Gene Ther. 6:457-467, 1995; Scaria et al., Gene Therapy 4:611-617, 1997).

Modifications to the adenovirus genome in the recombinant vector can decrease the host immune response (Yang et al., Nature Genetics 7:362-369, 1994; Lieber et al., J. Virol. 70:8944-8960, 1996; Gorziglia et al., J. Virol. 70:4173-4178; Kochanek et al., Proc. Natl. Acad. Sci. USA 93:5731-5736, 1996; Fisher et al., Virology 217:11-22, 1996). For example, the adenovirus E3 gp19K protein can complex with MHC Class I antigens and retain them in the endoplasmic reticulum, which prevents cell surface presentation and killing of infected cells by cytotoxic T-lymphocytes (CTLs) (Wold et al., Trends Microbiol. 437-443, 1994), suggesting that the presence of its encoding gene in a recombinant adenoviral vector may be beneficial.

The lack of persistent expression of adenoviral vector-delivered transgenes may also be due to limitations imposed by the choice of promoter and/or transgene contained in the transcription unit (Guo et al., Gene Therapy 3:802-801, 1996; Tripathy et al., Nature Med. 2:545-550, 1996. Further optimization of minimal adenoviral vectors for persistent transgene expression in target cells involves the synergistic choice of expression control elements and vector genome design such that expression is maximized and host immune response is limited (WO98/46780 Scaria et al., J.Virol. 72:7302-7309, 1998).

It is desirable to provide PAV with mimimal viral coding sequences that cannot elicit a strong host immune response, but which can take advantage of the ability of adenoviral vectors to deliver transgenes to a wide variety of target cells. Production of PAV requires the presence of adenovirus proteins in trans which facilitate the replication and packaging of a PAV genome into viral vector particles. Most commonly, such proteins are provided by infecting a producer cell with a helper adenovirus containing the genes encoding such proteins. However, such helper viruses are potential sources of contamination of a PAV stock during purification if they are able to replicate and be packaged into viral particles. It is advantageous, therefore, to increase the purity of a PAV stock by reducing or eliminating any production of helper viruses which can contaminate the preparation. Several strategies to reduce the production of helper viruses in the preparation of a PAV stock are disclosed in allowed U.S. Patent Application Serial No. 08/895,194 and U.S. Patent No. 5,670,488, issued September 23, 1997, incorporated

herein by reference. For example, the helper virus can contain mutations in the packaging sequence of its genome which prevent packaging, or may contain an oversized adenoviral genome which cannot be packaged due to size constraints of the virion.

Other strategies include the design of a helper virus with a packaging signal flanked by the excision target site of a recombinase, such as the cre-lox system (Parks et al., Proc. Natl. Acad. Sci.USA 93:13565-13570, 1996; Hardy et al., J.Virol. 71:1842-1849, 1997).

Detailed analysis of the structure of the adenovirus packaging signal has revealed that it is organized into a minimum of seven functional elements, identified as A repeats (Schmid et al., J. Virol. 71:3375-3384, 1997). Using this information, a PAV production system which comprises helper adenoviruses and producer cell lines optimized for PAV production is provided such that the packaging signal region is available for the production of a helper virus stock but is disabled during the production of a PAV vector stock. The present invention provides for an increased preferential packaging of PAVs in the production of purified vector stocks to further the development and widespread use of these vectors for gene transfer.

Summary of the Invention

The invention is directed to novel helper adenoviruses which facilitate the production and packaging of pseudoadenoviral vectors (PAV) by providing for the production of essential viral proteins in trans production and packaging required for PAV the helper viruses of the inventors are packaging defective due to the inclusion in the packaging signal region of their genomes of binding sequences for DNA binding and/or repressor proteins that prevent access by packaging proteins to this signal. The invention is also directed to to PAV producer cell lines expressing such nucleic acids encoding DNA binding and/or repressor proteins. The invention is further directed to methods for the production of PAVs with minimal contamination from helper viruses, using the helper viruses and producer cell lines of the invention.

Brief Description of the Drawings

Figure 1 shows a schematic diagram of strategies for the production of packaging-defective helper adenoviruses.

Figure 2 shows a schematic diagram of the FLP recombinase/FRT helper system for excision of packaging sequences from helper adenoviruses.

Figure 3a shows a schematic diagram of a helper adenovirus containing a packaging signal flanked by lambda operator binding sequences; Figure 3b shows the structure of the junction of the packaging signal and operator sequences.

Figure 4a shows a schematic diagram of a helper adenovirus containing a packaging signal flanked by lambda operator binding sequences; Figure 4b shows the structure of the junction of the packaging signal and operator sequences.

Figure 5 shows a schematic diagram of a helper adenovirus containing a packaging signal flanked by lambda operator binding sequences.

Figure 6 shows a schematic diagram of a helper adenovirus containing a packaging signal flanked by lambda operator binding sequences.

Figure 7 shows a schematic diagram of a series of helper adenoviruses which contain a packaging signal flanked by FRT binding sequences.

Figure 8 shows a schematic diagram of Ad2HelpFRT containing a packaging signal flanked by FRT binding sequences.

Figure 9 shows a schematic diagram of Ad2HelpFRTluc containing a packaging signal and luciferase marker gene flanked by FRT binding sequences.

Figure 10 shows a schematic diagram of helper adenovirus TBTP.

Figure 11 is a schematic diagram of a helper adenovirus with internal ITR sequences facilitating virus replication.

Figure 12 shows a schematic diagram of plasmid pTRE/FLPe6.

Figure 13a shows a schematic diagram of a tetracycline induction system for FLPe6 expression; Figure 13b shows tetracycline induction system for FLPe6 expression in 293-tet-OFF cells.

Figure 14 shows a schematic diagram of plasmid pTRE/FLPe6.

Figure 15 shows a schematic diagram of plasmid pCEP/FLPe6.

Figure 16 shows a schematic diagram of plasmid pOG-FLPe6.

Detailed Description of the Invention

The invention is directed to novel helper adenoviruses which facilitate the production and packaging of pseudoadenoviral vectors (PAV) by providing for the production of essential viral proteins in trans required for PAV production and packaging. The helper viruses of the invention are packaging defective due to the inclusion in the packaging signal region of their genome of binding sequences for DNA binding and/or repressor proteins that either prevent access by packaging proteins to this signal region or which facilitate enzymatic excision of the packaging signal. The invention is also directed to to PAV producer cell lines expressing nucleic acids encoding such DNA binding and/or repressor proteins. The invention is further directed to methods for the production of PAV with minimal contamination from helper viruses, using the helper viruses and producer cell lines of the invention.

A helper virus of the invention is defined as an adenovirus which is able to supply the viral proteins required in trans for the production of PAV or other minimal adenoviral vectors. In accordance with the invention, the helper virus genome is disabled for packaging, thereby allowing for preferential packaging of the PAV genomes into viral particles. The helper virus genome comprises at least those genes and/or regions of the adenovirus genome that are required to produce the viral proteins required in trans for the production of PAV. The adenovirus proteins supplied by the helper virus include inter alia the regulatory proteins from the adenovirus early (E) genomic regions, the capsid proteins encoded by the viral late (L) genomic regions, and other structural and non-structural proteins. The production of the proteins encoded by a helper virus genome facilitates the replication of the PAV genome during the production of vector stock. The adenovirus genes required in trans are not limited by virus serotype, and the helper viruses of the invention can contain adenovirus genes from more than one serotype. Structural proteins, which are supplied by the helper virus, can therefore be chosen so that the capsid proteins are derived from a desired serotype or serotypes and optimized for a particular use.

The helper virus genome is desirably modified according to the present invention such that packaging of helper virus particles is impaired or eliminated. Such disability

reduces or eliminates the production of helper virus in the preparation of a PAV stock, while allowing the helper virus itself to be propagated during the separate production of a helper virus stock. A helper virus of the invention is rendered packaging-defective by incorporation into its genome of a reduced length packaging signal or the insertion therein of heterologous nucleotide sequences (defined as binding sequences) into in or near the packaging signal region of the helper virus genome. Such binding sequences are capable of binding to specific DNA binding and/or repressor proteins, thereby either blocking the utilization of the cis-acting packaging signal (packaging-signal masked) or causing excision of the signal from the helper virus genome (packaging-signal deleted) (Figure 1).

A binding protein is defined herein as any protein or peptide (1) which is capable of binding to the binding sequences inserted into or near the packaging signal region of a helper virus genome so as to prevent utilization of the signal and repress packaging or (2) which is capable of binding to and induce cleavage at specific target sites.

Binding sequences are defined as nucleotide sequences inserted in proximity to, adjacent to or into the packaging signal region of the helper virus genome which are capable of binding to specific DNA binding and/or repressor proteins. A binding sequence of the invention, alone or in combination or tandem with other binding sequences, is capable of avidly binding one or more DNA binding and/or repressor proteins such that the packaging signal of a helper virus genome cannot be accessed by the viral packaging proteins or becomes excised from the genome. Preferably, the binding sequences interact with and bind DNA binding and/or repressor proteins. The binding sequences which are incorporated into or near or near the packaging signal region of the helper virus genome can be of various lengths, e.g., from about 8-30 nucleotides, but can also be tandem arrays of such sequences.

Preferred specific binding sequences of the invention include, but are not limited to, those derived from the bacteriophage lambda operator (Ptashne, M. A Genetic Switch, Cell Press and Blackwell Scientific Publications, 1986) and the papillomavirus E2 binding sequence (McBride et al., J.Biol. Chem. 266:18411-18414, 1991; Androphy et al., Nature 235:70-73, 1987). Most preferably, the packaging signal region in a helper

virus is flanked by the recognition site for a FLP recombinase, an enzyme which recognizes the FLP recombination target (FRT) and can catalyze site-specific excision of flanked nucleotide sequences (Senecoff et al., Proc. Natl. Acad.Sci. 82:7270-7274, 1985; O'Gorman et al., Science 251:1351-1355, 1991). Upon recognition of the FRT nucleotide sequences by a FLP recombinase, the flanked packaging signal is excised from the helper virus genome, thereby preventing the packaging of the helper virus genome and the production of helper virus particles (Figure 2). In a preferred embodiment of the invention, the FLP recombinase is used in a producer cell of the invention for enzymatic cleavage of the packaging signal in a helper virus because it exhibits increased thermostability at 37°C (Buchholz et al., NAR 24:4256-4262, 1996; Buchholz et al., Nature Biotech. 16:657-662, 1998). The invention contemplates the use of an FLP recombinase which is optimized for particular uses as needed in the form of monomers, dimers, tetramers or other multimeric forms.

Specific binding sequences which can be inserted in to an adenovirus genome at a site located in or near the adenovirus packaging signal region to accomplish the goals of the invention include the following:

Lambda operator binding sequences (Ptashne, M. A Genetic Switch, Cell Press and Blackwell Scientific Publications, 1986):

O_L1: TATCACCGCCAGTGGTA (SEQ ID NO:1)
ATAGTGGCGGTCACCAT

O_R1: TATCACCGCCAGAGGTA (SEQ ID NO:2)
ATAGTGGCGGTCTCCAT

O_L2: TATCTCTGGCGGTGTTG (SEQ ID NO:3)
ATAGAGACCGCCACAAC

O_L3: TATCACCGCAGATGGTT (SEQ ID NO:4)
ATAGTGGCGTCTACCAA

O_R2: TAACACCGTGCGTGTTG (SEQ ID NO:5)
ATTGTGGCACGCACAAC

O_R3: TATCACCGCAAGGGATA (SEQ ID NO:6)
ATAGTGGCGTTCCCTAT

In a preferred embodiment, the binding sequence is O_L1:

TATCACCGCCAGTGGTA (SEQ ID NO:1)
ATAGTGGCGGTCACCAT

Specific binding sequences for use by the papilloma virus E2 proteins include:

ACCGAAATCGGT (SEQ ID NO:7) Romanczuk et al., J.Virol. 64:2489-2859, 1990

ACCGAAACCGGT (SEQ ID NO:8) Romanczuk et al., J.Virol. 64:2489-2859, 1990

ACCN(6)GGT (SEQ ID NO:9) Androphy et al., Nature 325:70-73, 1985

Other papilloma virus E2 protein consensus sequences which can be used as binding sequences in accordance with the present invention are those described in McBride et al., J.Biol. Chem. 266:18411-18414, 1991.

The binding sequences responsive to the FLP recombinase include its recognition site, FLP recombinase target (FRT) repeated motifs underlined) (Senecoff et al., Proc.Natl.Acad.Sci. USA 82:7270-7274, 1985):

FRT: GAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC (SEQ ID NO:10)

Another binding site for the FLP recombinase is:

CGAAGTTCCTATTCTCTAGAAAGtATAGGAACTTC (SEQ ID NO:11)

The FRT binding sequences can be derived from plasmid pOG45 (Stratagene, La Jolla,CA) or can be synthesized using standard techniques for oligonucleotide synthesis. Truncated FRT sequences which also facilitate cleavage of an adjacent packaging signal in a helper virus are also within the scope of the invention. The use of any binding sequences which facilitate FLP-mediated excision of a packaging signal from a helper adenovirus is within the scope of the invention in the construction of packaging-defective helper adenoviruses.

Locations for the insertion of the binding sequences within or near a packaging signal in an adenovirus genome region can be identified by reference to the nomenclature

of the packaging signal region which defines a minimum of seven AT-rich elements, denoted AI-AVII. These seven AT-rich elements are located in the adenovirus genome from nucleotides 194-380 (referencing adenovirus serotype 5, Schmid et al., J. Virol. 71:3375-3384, 1997). For example, one or more binding sequences can be inserted into one or more sites flanking the A elements, or can be inserted into or between the A repeats. The redesign of the adenovirus packaging signal region according to the present invention also contemplates the deletion or multiplication of one or more A repeat elements. It will be evident to the skilled artisan that strategies for adenovirus genome design, which include various combinations of insertions of binding sequences or in close proximity or adjacent to the A repeats as well as deletions or repetitions thereof can accomplish the goal of the invention, i.e., disabling the packaging signal of the helper virus genome. Where the binding sequences facilitate the excision of a packaging signal, they may be inserted into the adenovirus genome in order to flank a desired packaging signal region at the 5' and 3' ends thereof, such that the entire signal region is excised by a site-specific recombinase.

In one preferred embodiment of a helper virus packaging signal, the helper virus genome is modified by the deletion of packaging elements AI-AIV, retaining only the packaging elements AV, AVI and AVII. A binding sequence comprising a 17 bp bacteriophage lambda operator sequence (preferably, O_L1) is inserted into the helper genome upstream and downstream from elements AV and AVII (sites #1 and #2), adjacent to nucleotides 334 and 385 of the adenovirus genome (Figure 3:Ad(AV-VI-AVII). Alternatively, the A repeat elements AV, AVI and AVII can be repeated as a motif, e.g., $(AV\text{-}AVI\text{-}AVII)_2$ and flanked by inserted lambda operator sequences as shown in Figure 4:$Ad(AV\text{-}AVI\text{-}AVII)_2$. Helper vectors Ad(AV-AVI-AVII) and $Ad(AV\text{-}AVI\text{-}AVII)_2$ can be used directly as helpers [packaging impaired, in the case of Ad(AV-AVI-AVII)] or in conjunction with lambda repressor, in a cell line expressing a nucleic acid encoding that protein (packaging-masked helper vector).

In another preferred embodiment, the packaging signal region of a helper virus genome is modified by the deletion of packaging elements AI-AII-AIII-AIV, retaining only the packaging elements AV, AVI and AVII. A binding sequence comprising a 17

bp operator sequence (preferably, O_L1) from bacteriophage lambda is inserted into the helper virus genome between elements AV and AVI (site #3), as well as upstream of AV and downstream from AVII (sites #1 and #2) (Figure 5).

In a further preferred embodiment, the packaging signal region of a helper virus genome is modified by the deletion of packaging elements AVI and AVII. A binding sequence comprising a 17 bp operator sequence from bacteriophage lambda (preferably, O_L1) is inserted into the helper virus genome between AII and AIII (site #5), as well as upstream from AI and downstream from AV (sites #4 and #6) (Figure 6).

Preferred sites within the packaging signal region of the helper virus genome for insertion of any binding sequences (with reference to the A repeats numbered from I-VII) include, but are not limited to:

Table 1

Site	Location relative to A repeat
1	5' to AV
2	3' to AVII
3	Between AV and AVI
4	5' to AI
5	Between AII and AIII
6	3' to AV

In another embodiment of a helper virus, binding sequences that bind, papilloma virus E2 protein are inserted into the helper virus genome to flank all or part of an packaging signal region in order to bind the E2 protein. Insertion sites set forth in Table 1 are preferred.

Where the binding sequences comprise a lambda operator sequence used with the lambda repressor to prevent the packaging of the helper virus during the production of PAV, it is preferable to flank the packaging signal region with the operator sequences, since cooperative binding of tetramers to pairs of operator sites is required for full repression (Ptashne, M. A Genetic Switch, Cell Press and Blackwell Scientific Publications, 1986). Prior studies have demonstrated the feasibility of blocking cis-acting signals using the lambda operator/repressor system, e.g., the TATA transcriptional element (Wedler et al., Mol. Gen. Genet. 248:499-505, 1995).

Where the binding sequences comprise a FRT binding sequence, specific designs of the packaging signal region of a helper virus genome (with reference to the A repeats I-VII) include, but are not limited to (Figure 7):

FRT(V-VI-VII)FRT

FRT(V-VI-VII)$_2$FRT

FRT(V-VI-VII)$_3$FRT

FRT(VI)$_{12}$FRT

FRT(VI)$_{12}$lucFRT

A preferred embodiment of a helper adenovirus which comprises the packaging signal regions FRT(VI)$_{12}$FRT or FRT(V-VI-VII)$_2$FRT includes Ad2HelpFRT (Figure 8).

It will be recognized by those skilled in the art that other combinations of deletions and insertions into and in proximity to the packaging signal region of a helper virus genome are within the scope of the invention and will create a helper virus which is disabled for packaging PAV in a cell line of the invention. Where a binding sequence is denoted (X) and an A repeat is denoted by a Roman numeral from I through VII and a subscript number denotes the number of times such a motif is repeated, other recombinant packaging signal regions include the following:

XVXVIXVII

XIXIIXIIIXIVXVXVIXVIIX

X(I-II-III-IV-V-VI-VII)X

X(I-II-III-IV-V-VI-VII)$_2$X

X(I-II-III-IV-V-VI-VI)$_3$X

X(I-II-III-IV-V)X

X(I-II-III-IV-V)$_2$X

X(I-II-III-IV-V)$_3$X

X(V-VI-VII)X

X(V-VI-VII)$_2$X

X(V-VI-VII)$_3$X

X(VI)$_{12}$X

X[(VI)$_{12}$]$_2$X

$X[(VI)_{12}]_3X$

Another aspect of the invention is directed to helper viruses which are designed to reduce or prevent recombination between nucleotide sequences in a helper virus packaging signal region and the PAV or between a packaging signal region or other regions in a helper virus genome and the adenovirus genome sequences in a packaging cell line, e.g., E1 sequences in 293 cells (nucleotides 1-4344 of adenovirus serotype 5), so as to prevent the generation of viable helper adenoviruses upon recombination.

Recombination between replication-defective adenoviral vectors and 293 cells leading to the generation of replication-competent adenoviruses has been demonstrated (Hehir et al., J.Virol. 70:8459-8467, 1996). Where a helper virus genome comprises binding sequences that flank the packaging signal region (e.g., lox sites which can be cleaved by the cre enzyme provided by a packaging cell line, Parks et al., Proc.Natl.Acad.Sci.USA 93:13565-13570, 1996, or contain the FRT target sites for the FLP recombinase), recombination between homologous nucleotide sequences in the helper virus genome and a producer cell could lead to the deletion of the flanking target sequences, e.g, the lox sites or the FRT sites. Such a deletion would therefore prevent the FLP or cre-mediated excision of the packaging signal in the helper virus which reduces packaging of the helper virus genomes during PAV production and packaging.

Accordingly, therefore, the invention also provides helper adenoviruses contain packaging signal regions that are not homologous with a PAV genome. In a particular embodiment of this aspect of the invention, a helper virus genome is provided which contains one or more AVI packaging signal sequences (reference for signal elements, Schmid et al., J. Virol. 71:3375-3384, 1997) which only minimally overlap with any nucleotide sequences in a PAV vector (e.g., nucleotides 1-356, containing only packaging signal elements AI-AV; disclosed in allowed U.S. Patent Application Serial No. 08/895,194 and U.S. Patent No. 5,670,488). The possibility of recombination between the PAV and the helper virus genome, therefore, is significantly reduced.

In a particularly preferred embodiment of a helper virus genome, the signal region comprises a (12 x AVI repeats) nucleotide sequence, or contains tandem repeats of this

signal. Such helper virus genome contains no overlapping nucleotide sequences with a PAV genome, which utilizes a packaging signal containing nucleotides sequences from the A repeats I-V. Such a helper packaging signal region can be flanked by binding sequences that facilitate excision of the packaging signal by a recombinase (e.g., lox, FRT) or by binding sequences that facilitate binding to the packaging signal by a repressor protein (e.g., lambda repressor) in accordance with the invention.

A marker gene also may be inserted into a helper virus packaging signal region in order to act as a marker for the excision of the packaging signal by loss of the characteristic signal encoded by the marker gene, e.g., luciferase (assay kits available from Promega, Madison, WI). A preferred genome of a marker helper adenovirus is Ad2HelpFRTluc (Figure 9) which is constructed such that, upon excision of the FRT-flanked packaging signal by an FLP recombinase, the signal from a luciferase protein can no longer be detected. However, recombination with homologous nucleotide sequences in a producer cell line would also result in loss of the marker signal, although the packaging signal will not be deleted. Therefore, a marker helper adenovirus is therefore most optimally used in a producer cell line which does not contain nucleotide sequences which may generate recombination with the 5' region in a helper virus and confound interpretation of a marker assay for excision of a packaging signal by an FLP recombinase. For example, a marker helper adenovirus may be used in a cell line, such as PER.C6 cells (containing adenovirus serotype 5 nucleotides 459-3510), whose genome comprises adenovirus sequences which only minimally overlap with any 5' nucleotide sequences in a helper virus, thereby reducing the likelihood of recombination (Fallaux et al., Hum.Gene Ther. 1:1909-1917, 1998). PER.C6 cells are preferably used in the production methods of the invention in order to minimize recombination between producer cells and a helper adenovirus.

In a further embodiment of the invention, a helper virus is provided that reduces the possibility of recombination between the helper virus genome and the overlapping adenovirus nucleotide sequences in a complementing cell line (e.g., E1 sequences in 293 cells) or in a PAV that can result in the production of viable helper viruses which contaminate a PAV preparation. The genome of helper virus comprises a packaging

signal which is prevent in a reverse orientation such that, even though recombination may occur between the helper virus and either the packaging cell or the PAV genome, the resulting recombination products do not constitute viable adenovirus. The packaging signal of such helper virus comprises any combination of the A repeat elements (Schmid et al., J. Virol. 71:3375-3384, 1997) sufficient to confer packaging capability, inserted into the genome in a reverse orientation. In a particularly preferred embodiment, the packaging signal region includes a (12 x AVI repeats) sequence in a reverse orientation.

In a further embodiment of the invention, a helper virus is provided which is inactivated upon the acquisition of homologous sequences from a recombination event with a complementing cell line, preferably an E1-complementing cell line, such as 293 cells. For example, this helper virus is engineered such that its size, upon a recombination event, renders the virus genome too large for packaging. A preferred embodiment is the helper virus TBTP whose genome contains a FRT-flanked packaging signal (Figure 10), and in which the E3 region of the adenovirus genome is deleted for 2.9 kb, but into which the 1.8 kb EGFP gene operably linked to a CMV promoter and a 5.0 kb fragment of the human alpha-antitrypsin gene are inserted. The size of the helper virus is 36.9 kb (102% of wild-type), but upon recombination with adenovirus E1 sequences in 293 cells, the helper virus genome size becomes approximately 39.6 kb (110%), which is too large to be packaged.

To create the particular helper viruses of the invention, the adenovirus genome, whether wild-type or recombinant, is modified by the insertion thereto of binding sequences in proximity to or into the A repeats of the packaging signal region so as to prevent access to the cis-acting packaging signal upon the binding of a DNA binding and/or repressor protein to the specific binding sequences. Standard techniqes of molecular biology such as restriction enzyme digestion and ligation, polymerase chain reaction and site-directed mutagenesis can be used to create a recombinant packaging signal region within a helper adenovirus genome. Such a packaging signal region can then be inserted into the appropriate site in the 5' region of an adenovirus genome utilizing a plasmid comprising the signal. Such a plasmid can be co-transfected into a cell line with DNA encoding the remainder of the adenovirus helper genome to be contained in the

helper virus, such that homologous recombination occurs, thereby generating a helper adenovirus with the desired recombinant packaging signal. The helper viral genome can also be constructed in bacteria, thus simplifying the procedure (Chartier et al., J.Virol. 70:4805-4810, 1998). The entire viral genome can be provided by transfection of a plasmid from which any non-viral (i.e., bacterial) sequences have been removed. Other methods for the production of recombinant adenoviruses are known to those skilled in the art and can be used to produce the helper viruses of the invention.

The helper viruses of the invention can be derived from any wild-type, truncated or mutant adenovirus whose genome encodes the viral proteins required in trans to produce PAVs, and are not limited by serotype. Preferably, the helper viruses of the invention are also replication-defective, as an additional safety feature for the use in generating PAVs for use in gene transfer. Replication-defective viruses can be created by, for example, deletion of the E1 region of the adenovirus genome. Such helper viruses can be propagated in E1-complementing cell lines such as the 293 cell line (Graham et al., J.Gen.Virol.36:59-72, 1977).

Although the invention provides packaging-defective helper adenoviruses, the genomes of such viruses are desirably optimized for replication and gene expression in order to ensure adequate levels of adenoviral helper proteins. Therefore, in one particular embodiment of the invention, a helper virus genome contains an internal ITR sequence located downstream from the blocked packaging signal region which allows for adequate replication and expression of the helper virus genome in the PAV producer cell line, thereby ensuring adequate provision of the viral proteins required in trans. Although a repressor protein can occupy the binding sequences inserted into or near a helper virus packaging signal, this particular embodiment of the invention provides an exposed ITR which is available to the adenovirus DNA polymerase and terminal protein for replication of the helper virus genome (Figure 11).

In a further aspect of the invention, a method for the production of PAV vectors is provided which uses a PAV genome and a helper adenovirus that contain packaging signal regions or ITR sequences from different adenovirus serotypes such that sequence

overlap between PAV genome and helper adenovirus is minimized and the possibility of recombination is reduced.

The invention is further directed to PAV producer cell lines that produce DNA binding and/or repressor proteins which can bind to the binding sequences inserted into or near the packaging signal region of the helper virus genome. As a result of this interaction, the helper virus is disabled for packaging or deleted during the production of PAV stocks. In a preferred embodiment of the invention, 293 cells comprising and express a nucleic acid encoding a DNA binding and/or repressor protein, thereby creating a PAV producer cell line of the invention. Other cell lines can be used, including, but not limited to, VK2-20, as well as any cell lines designed to complement deletions of adenoviral genomic regions E2a (Zhou et al., J. Virol. 70:7030-7038, 1996), E4 (Krougliak et al., Hum. Gene Ther. 6:1575-1586, 1995; Wang et al., Gene Ther. 2:775-783, 1995), or protein IX (Caravokyri et al., J. Virol. 69:6627-6633, 1995; Krougliak et al., Hum. Gene Ther. 6:1575-1586, 1995).

Preferably, 293 cells comprise and express a nucleic acid encoding a DNA binding protein, preferably the FLP or FLPe recombinase, under the control of the tetracycline gene control system (Gossen and Bujard, Proc. Natl. Acad. Sci. USA 89:5547-5551, 1992; and Gossen et al., Science 268:1766-1769, 1995, both incorporated here by reference). In this system, gene expression from a minimal promoter is under strict control of the tetracycline repressor (TetR), expressed as a fusion protein with the herpes virus VP16 transcriptional activation domain (TetR/VP16). Two versions of the TetR/VP16 protein exist: the wild type TetR is active only in the *absence* of tetracycline or doxycycline, while a mutated form of TetR (reverse TetR or rTetR) is active only in the *presence* of doxycycline. When linked to VP16, the TetR form activates transcription only in the absence of tetracycline and the rTetR form activates transcription only in the presence of doxycycline. Each of these transcriptional control factors, TetR/VP16 and rTetR/vp16 controls the expression of genes linked to a minimal promoter cloned adjacent to tetracycline transcriptional regulatory elements (TRE), such as a gene for FLP recombinase stably integrated into 293 cells. A 293 cell line incorporating a nucleic acid encoding the Tet-off fusion protein (TetR-VP16) can be constructed by the manufacturer

(Clontech, Palo Alto, CA) or can be produced by transfection of a Tet-off plasmid available from Clontech. It will be noted that a 293-Tet-off cell line which further expresses a FLP recombinase can also be used to inducibly and preferentially excise a helper adenovirus packaging signal region which is flanked by FTR binding sequences or could also be used to excise any desired segment of an adenoviral genome which is flanked by the requisite FRT binding sequences.

In this embodiment of the invention, therefore, a nucleic acid encoding the FLP recombinase is cloned into a pTRE plasmid (Clontech, Palo Alto, CA) (Figure 12), such that the FLP recombinase is under the control of a minimal CMV promoter operably linked thereto and seven tet operator sites which are responsive to the TetR-VP16 fusion protein (Figure 13a). When tetracycline is provided to the 293 Tet-off producer cells, transcription from the FLP recombinase gene does not occur (Figure 13b). Modulation of expression of the FLP recombinase, and therefore of FLP-catalyzed excision of a helper adenovirus packaging signal in a dose-dependent manner can occur relative to the level of tetracycline provided to the producer cell.

Preferably, a producer cell line of the invention is a 293 cell line comprising a nucleic acid encoding Tet-off fusion protein and the FLP recombinase under the control of the TRE operator sequences and the minimal CMV promoter such that the cell line provides modulated production of the FLP recombinase when provided with a PAV genome and a helper virus of the invention containing a packaging signal flanked by FRT binding sequences, such that the helper virus is preferentially packaging-disabled during PAV vector production.

Producer cell lines which stably express a nucleic acid encoding the FLP recombinase can also be constructed using plasmids which contain the gene encoding FLP recombinase under the control of any suitable promoter, such as CMV or SV40. Examples of such plasmids which contain a FLP recombinase gene are pSVK/FLPe6 (containing FLP recombinase gene under the control of the SV40 promoter) (Figure 14) and pCEP/FLP36, containing FLP recombinase gene operably linked to the CMV promoter (Figure 15). Where it is desirable to provide the FLP recombinase gene to a cell

for immediate expression therein and assay, a plasmid which is maintained extrachromosomally, e.g., pCEP/FLPe6 (Figure 15) can be used.

Nucleic acids encoding the DNA binding and/or repressor proteins are engineered into PAV producer cell lines by standard techniques of molecular biology, and can be stably or inducibly expressed. Preferred DNA binding and/or repressor proteins to be used in the invention include, but are not limited to, the bacteriophage lambda repressor (wild-type and/or N-terminal fragment) Cro protein (Ptashne, M. A Genetic Switch, Cell Press and Blackwell Scientific Publications, 1986); the bovine papillomvirus E2 protein (McBride et al., J.Biol. Chem. 266:18411-18414, 1991), tetracycline repressor (Gossen et al., Proc.Natl.Acad.Sci. USA 89:5547-5551, 1992),tryptophan repressor, and others known to those skilled in the art. Specific recombinases of the invention which can be used to excise a packaging signal flanked by the appropriate binding sequences include, but are not limited to, FLP recombinase (Broach et al., Cell 21:501, 1980) or FLPe recombinase (Buchholz et al., NAR 24:4256-4262, 1996; Buchholz et al., Nature Biotech. 16:657-662, 1998). Other DNA binding and/or repressor proteins which are capable of binding to the relevant binding sequences in a helper virus genome can be used in the cell lines of the invention. Fusion proteins of such repressor proteins can also be constructed, for example, by creating a nucleic acid encoding E. coli β-galactosidase linked to the lambda repressor protein, thereby providing a substantial physical obstacle to packaging of the helper virus genome when such a fusion protein binds to the binding sequence inserted into or near a packaging signal in a helper virus genome. Fusion proteins can also be generated by creating a hybrid nucleic acid encoding a DNA binding and/or repressor protein fused to an activator protein (see tet repressor/HSV VP16, Gossen et al., Proc.Natl.Acad.Sci. USA 89:5547-5551, 1992).

The lambda repressor protein monomer is 236 amind acids in length (26KD); a repressor protein dimer binds to one 17 bp lambda operator sequence. The 6 lambda operator sites are recognized by the lambda repressor protein dimer in order of their intrinsic affinities for a lambda repressor dimer, each with a central base pair, the axis of symmetry. The N-terminal protein domain of the lambda repressor recognizes the operator and can be used for binding; in one embodiment of the invention, the C-terminal

domain of the repressor can be replaced with, for example, a dimeric leucine zipper protein.

To create the PAV producer cell lines of the invention, a nucleic acid encoding a DNA binding and/or repressor protein and the nucleotide sequences for any operably linked regulatory elements are introduced into a cell line by any method of nucleic acid transfer, including, but not limited to, transfection, electroporation, or viral-mediated transfer. A plasmid comprising a nucleic acid encoding a DNA binding and/or repressor protein and nucleotide sequences corresponding to any regulatory elements can be transfected into a cell of interest. If the plasmid further contains a nucleic acid encoding a selectable marker, integration of the exogenous plasmid DNA can be detected using such marker. For example, a nucleic acid encoding neomycin resistance can be introduced in parallel with the nucleic acid encoding a repressor protein, and the cells which are stably transfected thereby can be selected by cultivation in the presence of G418. Alternatively, a nucleic acid encoding such the repressor protein can be provided to a cell on an extrachromosomal plasmid which is maintained episomally (e.g., EBNA-based system), such as pCEP-4 (Invitrogen, San Diego, CA).

It is within the scope of the invention to use any binding sequence-repressor protein pair in the design of the helper viruses and cell lines of the invention which is able to effectuate a binding interaction that prevents utilization of the packaging signal in a helper virus genome or which facilitates the excision of the signal, thereby preventing packaging of the helper virus.

The helper adenoviruses and producer cell lines are useful in high-level production of PAV, allowing for preferential packaging of PAV genomes into gene transfer vectors relative to the helper viruses, thereby providing a means to produce helper-dependent PAVs with minimal contamination by helper viruses.

The invention is also directed to methods for the production of PAVs in high yield, using the helper viruses and producer cell lines of the invention. In such methods PAV is preferentially produced, generating an enriched preparation. To produce a PAV stock, the PAV genome which comprises the adenovirus 5' ITR and packaging signal and 3' ITR, and further comprises one or more transgenes up to 36 kb in size, operably linked

to expression control sequences, can be engineered into a plasmid using standard techniques of molecular biology (see e.g., Allowed U.S. Patent Application Serial No. 08/895,194 and U.S. Patent No. 5,670,488, incorporated herein by reference). The DNA fragment comprising the PAV genome is then enzymatically excised from the plasmid and co-transfected with a helper virus of the invention into a producer cell line of the invention. In accordance with the invention, PAVs are preferentially packaged because the PAV genome contains a wild-type packaging signal, in contrast to the helper virus in which the packaging signal has been disabled or deleted.

When the PAV genome is delivered to a producer cell on a plasmid, such plasmid can be introduced into a cell line of the invention by any method of nucleic acid transfer, including, but not limited to, transfection, lipofection and electroporation. The cell line can be infected with an adenovirus helper which is available to provide the adenovirus proteins needed in trans to produce and package the PAV genome. In a preferred embodiment of the invention, 2-20 μg of DNA which encodes a PAV genome is delivered to a cell by lipofection using a kit such as Profectin (Promega, Madison, WI), and the cells are infected with a helper virus of the invention using a multiplicity of infection (MOI) from 0.5 to 10.

In another aspect of the invention, a PAV producer cell line comprises an integrated PAV genome, as well as a nucleic acid encoding DNA binding and/or repressor protein, such that the PAV genome can be conditionally excised and the nucleic acid encoding the binding and/or repressor protein can be conditionally expressed. In a preferred embodiment, a PAV vector genome flanked by lox nucleotide sequences is stably integrated into a PAV producer cell line which is engineered to express a nucleic acid encoding the Cre recombinase (Sternberg et al., J.Mol. Biol. 150:467-486, 1981; Sauer et al., Meth. Enzymol. 225:890-910, 1993) and a repressor protein. Both Cre recombinase and the repressor protein can be inducibly produced using one or more inducible promoters susceptible to induction by such agents as tetracycline or ecdysone, among others. Upon infection by a helper virus of the invention, and induction of the recombinase and repressor, the PAV genome is excised and available for replication and packaging by the helper virus, while the helper virus is rendered packaging-disabled by

the repressor protein. This strategy for the production of PAV requires only infection of the producer cell by a helper virus and induction of the recombinase and repressor proteins to generate a preferentially packaged PAV stock. The use of other site-specific recombinases is also within the scope of this embodiment of the invention, e.g., the FLP recombinase and its target sequence, FRT.

Purification of PAVs from a cell line of the invention can be performed by standard techniques of virus purification known to those skilled in the art. For example, viruses in cell lysates from producer cells can be purified on a standard CsCl gradient. The PAV particles are of lower density relative to the helper viruses and will band at a higher position in the gradient, allowing for direct isolation and recovery. Alternatively, PAV purification can be performed using chromatographic techniques, e.g., as set forth in published PCT Application WO97/08298, incorporated herein by reference.

PAV yield is calculated by measuring the DNA and protein composition of the purified preparation. Maizel et al. (Virology 36:115-125, 1968) determined that an adenovirus virion comprises 13% DNA, with the remainder being protein.

Transgene activity of a PAV preparation is monitored by immunoflourescent techniques by infecting 293 cells with the PAV helper, then using an antibody against a PAV-encoded transgene expression product (protein) to determine infectious particles. Alternatively, enzyme activity encoded by a transgene can be measured (e.g., β-galactosidase, α-galactosidase, α-antitrypsin), by, for example, an ELISA assay. The ability of PAV to enter cells is determined by measuring the amount of viral capsids that bind to the cells with anti-adenovirus antibodies. Demonstration of the entry of the PAV genome into a cell can be performed by fluorescent in situ hybridization (FISH).

Minimal contamination of the PAV stock is expected using the novel helper viruses of the invention. Helper virus production, if any, can be scored, for example, by standard plaque assays on 293 cells. Preferably, the ratio of PAV to helper virus will be greater than 10,000 to 1.

The practice of the invention employs, unless otherwise indicated, conventional techniques of protein chemistry, molecular virology, microbiology, recombinant DNA technology, and pharmacology, which are within the skill of the art. Such techniques are

explained fully in the literature. See, e.g., Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons, Inc., New York, 1995, and Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, PA, 1985.

The invention is further illustrated by the following specific examples which are not intended in any way to limit the scope of the invention.

EXAMPLE 1: Construction of FRT-containing helper adenoviruses

Construction of pAD/FRT(AV-AVI-AVII)$_2$FRT and pAD/FRT(AV-AVI-AVII)$_3$FRT. Plasmid pAd/ITR (1-194)Mlu2 was digested with SpeI and Mlu I. Oligonucleotides containing a 5'→3' FRT site upstream from two copies of the AV-AVI-AVII packaging sequence were annealed together and ligated into the SpeI/Mlu I site of pAD/ITR(1-194)Mlu 2. The resulting vector was designated pAD/FRT(AV-AVI-AVII)^2. Oligonucleotides 4758 (5' - CGC GTG AAG TTC CTA TTC CGA AGT TCC TAT TCT CTA GAA AGT ATA GGA ACT TCA) (SEQ ID NO:12) and 4759 (5' - CGC GTG AAG TTC CTA TAC TTT CTA GAG AAT AGG AAC TTC GGA ATA GGA ACT TCA) (SEQ ID NO:13), containing a second FRT site oriented in the same direction as the first, were annealed together and ligated into the Mlu I site of pAD/FRT(AV-AVI-AVII)$_2$ and designated pAD/FRT(AV-AVI-AVII)$_2$FRT (Figure 7). Alternatively, oligonucleotides 4760 (5' - CGC GTC GCG TAA TAT TTG TCT AGG GCC GCG GGG ACT TTG ACC GTT TAG AAG TTC CTA TTC CGA AGT TCC TAT TCT CTA GAA AGT ATA GGA ACT TCA) (SEQ ID NO:14) and 4761 (5' - CGC GTG AAG TTC CTA TAC TTT CTA GAG AAT AGG AAC TTC GGA ATA GGA ACT TCT AAA CGG TCA AAG TCC CCG CGG CCC TAG ACA AAT ATT ACG CGA) (SEQ ID NO:15), containing a third copy of AV-AVI-AVII upstream from a second FRT site, were annealed together and ligated into the Mlu I site of pAD/FRT (AV-AVI-AVII)$_2$ and designated pAD/FRT(AV-AVI-AVII)$_3$FRT (Figure 7).

Construction of pAD/FRT(AVI)$_{12}$/luc/FRT using oligonucleotides containing twelve copies of AVI. Oligonucleotides containing a 5'→3' FRT site upstream from twelve head-to-tail copies of the AVI packaging sequence were annealed together and ligated into the Spe I / Mlu I site of pAD/ITR(1-194)Mlu2, generating pAD/FRT(AVI)$_{12}$ pGL3 control vector (Promega, Madison, WI) was digested with Mlu I and Bam HI and the 2427 bp fragment containing the luciferase cDNA under the control of the SV40 promoter/enhancer elements was isolated. Oligonucleotides containing a second FRT site were annealed together and coligated with a 2427 bp luciferase fragment into the Mlu I site of pAD/FRT(AVI)$_{12}$. The resulting vector containing an FRT flanked (AVI)$_{12}$/luc cassette was designated pAD/FRT(AVI)$_{12}$/luc/FRT (Figure 9).

Construction of pAD/FRT(AVI)$_{12}$FRT using an oligonucleotide containing one copy of AVI. Head-to-tail copies of the AVI packaging sequence were constructed by concatomerizing oligonucleotides 4755 (5' - TCG ACC GCG GGG ACT TTG ACC) (SEQ ID NO:16) and 4754 (5' - TCG AGG TCA AAG TCC CCG CGG) (SEQ ID NO:17) in the presence of T4 DNA ligase. Following ligation, the reaction was digested with Xho I to eliminate head-to-head and tail-to-tail ligation products, and cloned into the Xho I site of pSL1180 (Amersham Pharmacia Biotech, Piscataway, NJ) generating pSL/(AVI)$_{12}$ FRT sites flanking the AVI repeats were inserted in two consecutive cloning steps. pSL/(AVI)$_{12}$ was digested with Spe I and partially digested with Xho I. Oligonucleotides HR100 (5' - CTA GTG AAG TTC CTA TTC CGA AGT TCC TAT TCT CTA GAA AGT ATA GGA ACT TCC) (SEQ ID NO:18) AND HR101 (5' - TCG AGG AAG TTC CTA TAC TTT CTA GAG AAT AGG AAC TTC GGA ATA GGA ACT TCA) (SEQ ID NO:19) containing an FRT site was ligated in to the Spe I / Xho I sites upstream from (AVI)$_{12}$. The resulting vector, pSL/FRT(AVI)$_{12}$ was digested with Mlu I and partially digested with Xho I. Oligonucleotides HR102 (5' - TCG AGG AAG TTC CTA TTC CGA AGT TCC TAT TCT CTA GAA AGT ATA GGA ACT TCA) (SEQ ID NO:20) and HR103 (5' - CGC GTG AAG TTC CTA TAC TTT CTA GAG AAT AGG AAC TTC GGA ATA GGA ACT TCC) (SEQ ID NO:21) containing a second FRT site was ligated into the Spe I /Xho I site downstream from (AVI)$_{12}$. The resulting vector was designated pSL/FRT(AVI)$_{12}$FRT.

Helper adenoviruses containing the above-described designs of the 5' adenovirus packaging signal region are constructed in vitro using standard ligation techniques or by homologous recombination in vivo with any desired adenovirus.

EXAMPLE 2: Construction of helper adenoviruses containing lambda operator sites

Helper vector Ad(AV-AVI-AVII). Adenovirus nucleotides 1 through 194 were isolated by PCR and cloned into pAdvantage (Genzyme Gene Therapy, Framingham MA), in place of Ad nucleotides 1 through 357. pAdvantage encodes the Ad2 genome (ΔE1, E3Δ2.9) in pBR322. Sequences encoding a deleted packaging site (AV-AVI-AVII) (nucleotides 334-385) were cloned into an SpeI / MluI site within the vector and flanked

by binding sites for the lambda repressor (Figure 3a; junction sequences are shown in Figure 3b). This vector is 40-fold impaired in packaging, compared to a wild-type vector.

Helper vector Ad(AV-AVI-AVII)$_2$ was built similarly to Ad(AV-AVI-AVII) (Figure 4a; junction sequences are shown in Figure 4b). This helper vector incorporates 2 copies of the (AV-AVI-AVII) packaging repeat sequences, giving it a packaging efficiency equivalent to wild-type

EXAMPLE 3: Construction of TBTP helper adenovirus

The E3 gene of Ad2 (nts 27970 - 30937) is replaced with a 1.8kb EGFP expression cassette (CMV / EGFP / SvpA), at a genetically engineered RsrII site in the E3-deleted genome. A 5.0 kb Hinp1I/AccI fragment from the human genomic AAT gene (ghAAT) is cloned in an AccI site just upstream of the EGFP expression cassette. The size of the completed vector is 36.9 kb (102% wild-type) (Figure 10). After recombination with adenovirus sequences from 293 cells, the vector size will be approximately 39.6 kb (110%).

EXAMPLE 4: Construction of producer cells expressing FLP recombinase

Plasmids

pFLPe6. pOG-Flpe6 was received from Francis Stewart (EMBL, Heidelberg, Germany) (Figure 16). This gene contains mutations of the Flp gene that make the encoded protein more stable at 37°C. The gene, with upstream and downstream regulatory elements, is cloned into pOG44 (Stratagene, La Jolla, CA) behind the CMV promoter (Figure 16).

pSVK/FLPe6. The 2.0 kb XbaI / SalI fragment of pOG-Flpe6 was cloned directly into the XbaI / SalI site of the 3.9 kb pSVK3 (Pharmacia, Piscataway, NJ). The plasmid pSVK/FLPe6 has the Flpe6 gene under the control of both eukaryotic (SV40e) and Prokaryotic (T7) promoters (Figure 14).

pCEP/FLPe6. The 2.0 kb XbaI / SalI fragment of pOG-Flpe6 was cloned into the Acc65I / XhoI site of the 10.4 kb pCEP-4 (Invitrogen, San Diego, CA) using adapter

linkers. The plasmid pCEP/FLPe6 has the Flpe6 gene under the control of the CMV promoter. The plasmid retains the EBNA-1 gene and EBV origin for extrachromosomal plasmid maintenance and the hygromycin gene for plasmid selection (Figure 15).

pTRE/FLPe6. The 2.0 kb XbaI / SalI fragment of pOG-Flpe6 was cloned into Acc65I / XhoI site of the 3.1 kb pTRE plasmid (Clontech, Palo Alto, CA) using adapter linkers. The Flpe6 gene in pTRE/FLPe6 is under the control of a minimal CMV promoter (hCMV*) and 7 operator sites (tet O, 1 through 7).

Producer Cells

pCEP/Flpe6 cells. 293 and PER.C6 cells are transfected with 10-20μg of pCEP/Flpe6 DNA. Following hygromycin selection, cells expressing high levels of Flpe6 are selected based on a functional assay for FLP activity, such as FRT-mediated excision of a target sequence. Cells with a stable extrachromosomal plasmid copy number are selected for PAV amplification.

pSVK/Flpe6 cells. Cells are transfected similarly as described above, with the addition of a plasmid bearing a neo resistance gene marker. Following neo selection, cells expressing high levels of Flpe6 are selected based on a functional assay for FLP activity, such as FRT-mediated excision of a target sequence. Cells with stable, integrated pSVK/Flpe6 DNA are selected for PAV amplification.

pTRE/Flpe6 cells. Cells are transfected as described above, with the addition of the pTK/hygro plasmid (Clontech, Palo Alto, CA) in the transfection. Following hygromycin selection, cells expressing low background and high tetracyline-induced levels levels of Flpe6 are selected based on a functional assay for FLP activity, such as FRT-mediated excision of a target sequence. Cells with stable, integrated, inducible pTRE/Flpe6 are selected for PAV amplification.

EXAMPLE 5: Propagation of PAV using helper adenoviruses

PAV DNA excised from backbone DNA (4-20μg) is used to transfect semi-confluent PER.C6 or 293 cells. To select for PAV packaging at the expense of helper vector, the cells express a repressor protein, which, upon binding to cognate sequences in

the helper vector, prevents its packaging. After overnight incubation, cells are infected at an MOI of 1 to 10 with an E1-deleted helper adenovirus harboring the appropriate packaging impaired sequences. Following the observation of complete cytopathic effect, cells and lysates are collected and the lysate is used for serial propagayion and expansion of PAV. Following several amplifications, the cell lysate is CsCl banded for the isolation of PAV.

Claims

1. A helper adenovirus which facilitates the production of pseudoadenoviral vectors comprising an adenvoirus genome into which is inserted into or in proximity to the packaging signal region of the genome a nucleic acid or nucleic acid comprising binding sequences for a DNA binding and/or repressor protein, wherein binding of said protein to said binding sequences prevents packaging of the helper virus.

2. The helper adenovirus of Claim 1, in which the binding sequences comprise a nucleic acid encoding a lambda operator sequence.

3. The helper virus of Claim 2, in which the binding sequences comprise SEQ. ID NO.1.

4. The helper adenovirus of Claim 1, in which the binding sequences comprise an FLP recombination target sequence (FRT) recognized by an FLP recombinase.

5. A helper adenovirus which facilitates the production of pseudoadenoviral vectors comprising an adenovirus genome wherein said genome comprises a packaging signal region having an AV-AVI-AVII packaging element repeat sequence.

6. A helper adenovirus which facilitates the production of pseudoadenoviral vectors comprising an adenovirus genome wherein said genome comprises a packaging signal region having two copies of an AV-AVI-AVII packaging element repeat sequence.

7. A helper adenovirus which facilitates the production of pseudoadenoviral vectors comprising an adenovirus genome wherein said genome comprises a packaging signal region having three copies of an AV-AVI-AVII packaging element repeat sequence.

8. A helper adenovirus which facilitates the production of pseudoadenoviral vectors comprising an adenovirus genome wherein said genome comprises a packaging signal region having twelve copies of an AVI packaging element repeat sequence.

9. The helper adenovirus of Claim 5, further comprising a nucleic acid comprising lambda operator binding sequences inserted into the adenovirus genome at a location flanking the packaging signal.

10. The helper adenovirus of Claim 5, further comprising a nucleic acid comprising an FLP recombination target sequence (FRT) into the adenovirus genome at a location flanking the packaging signal.

11. The helper adenovirus of Claim 6, further comprising a nucleic acid comprising lambda operator binding sequences inserted into the adenovirus genome at a location flanking the packaging signal.

12. The helper adenovirus of Claim 6, further comprising a nucleic acid comprising an FLP recombination target sequence (FRT) into the adenovirus genome at a location flanking the packaging signal.

13. The helper adenovirus of Claim 8, further comprising a nucleic acid comprising lambda operator binding sequences inserted into the adenovirus genome at a location flanking the packaging signal.

14. The helper adenovirus of Claim 8, further comprising a nucleic acid comprising an FLP recombination target sequence (FRT) into the adenovirus genome at a location flanking the packaging signal.

15. A producer cell line for the production of pseudoadenoviral vectors, comprising and expressing a stably integrated nucleic acid encoding a DNA binding and/or repressor protein.

16. The producer cell line of Claim 15, in which the DNA binding protein is the lambda repressor.

17. The producer cell line of Claim 15, in which the DNA binding protein is an FLP recombinase.

18. The producer cell line of Claim 15, wherein the cell line is the 293 cell line.

19. The producer cell line of Claim 15, wherein the cell line is the PER.C6 cell line.

20. The producer cell line of Claim 18, in which a nucleic acid encoding TetR/VP16 operably linked to expression control sequences is stably integrated into the genome of the cell line.

21. The producer cell line of Claim 20, further comprising a stably integrated nucleic acid encoding an FLP recombinase operably linked to expression control sequences comprising tetracycline transcriptional regulatory elements (TRE).

22. A method for the production of pseudoadenoviral vectors, comprising transfecting a producer cell line comprising and expressing a stably integrated nucleic acid encoding an FLP recombinase operably linked to expression control sequences with a pseudoadenoviral vector genome (PAV) and a helper adenovirus comprising an adenovirus genome into which binding sequences comprising an FLP recombination target sequence (FRT) sequence has been inserted in a location flanking the packaging signal, whereby the packaging signal of the helper

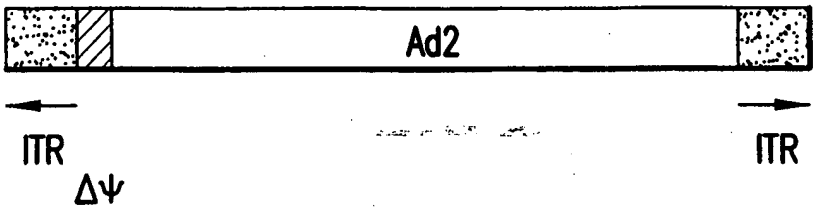
adenovirus is excised by the FLP recombinase, and isolating the PAV vectors produced in said cell line.

23. The method of Claim 22, wherein said PAV genome comprises an adenovirus genome comprising 5' and 3' ITR sequences, an adenovirus packaging signal and one or more transgenes up to 36 kb in size operably linked to expression control sequences.

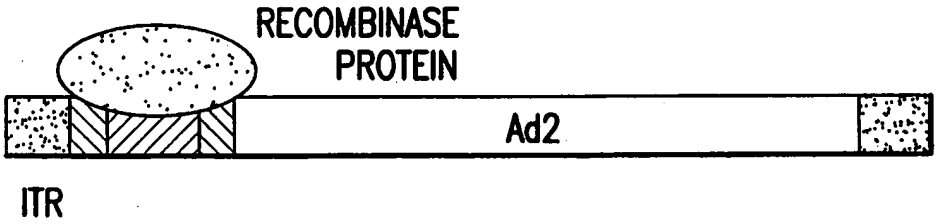
24. A method for the production of pseudoadenoviral vectors, comprising transfecting producer cell line comprising a stably integrated nucleic acid encoding the TetR/VP16 fusion protein operably linked to expression control sequences and further comprising a stably integrated nucleic acid encoding an FLP recombinase operably linked to expression control sequences comprising tetracycline transcriptional regulatory elements (TRE) with a pseudoadenoviral vector (PAV) genome and a helper adenovirus comprising an adenovirus genome into which binding sequences comprising an FLP recombination target sequence (FRT) sequence has been inserted in a location flanking the packaging signal, whereby the packaging signal of the helper adenovirus is excised by the FLP recombinase, and isolating the PAV vectors produced in said cell line.

25. The method of Claim 24, wherein said PAV genome comprises an adenovirus genome comprising 5' and 3' ITR sequences, an adenovirus packaging signal and one or more transgenes up to 36 kb in size operably linked to expression control sequences.

PACKAGING IMPAIRED HELPER VECTOR



PACKAGING-SIGNAL DELETED HELPER VECTOR



PACKAGING-SIGNAL MASKED HELPER VECTOR

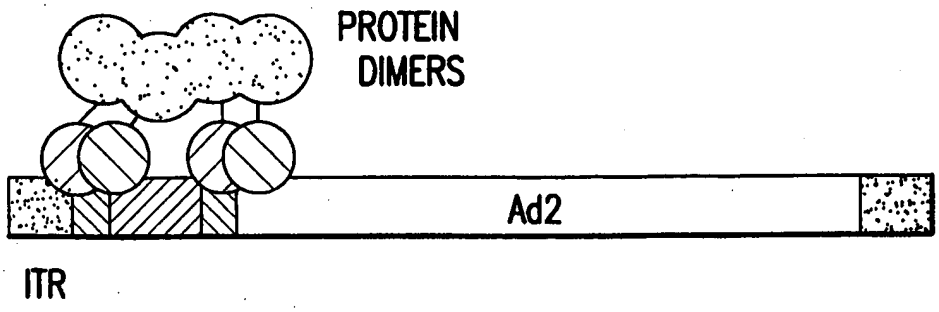


FIG.1

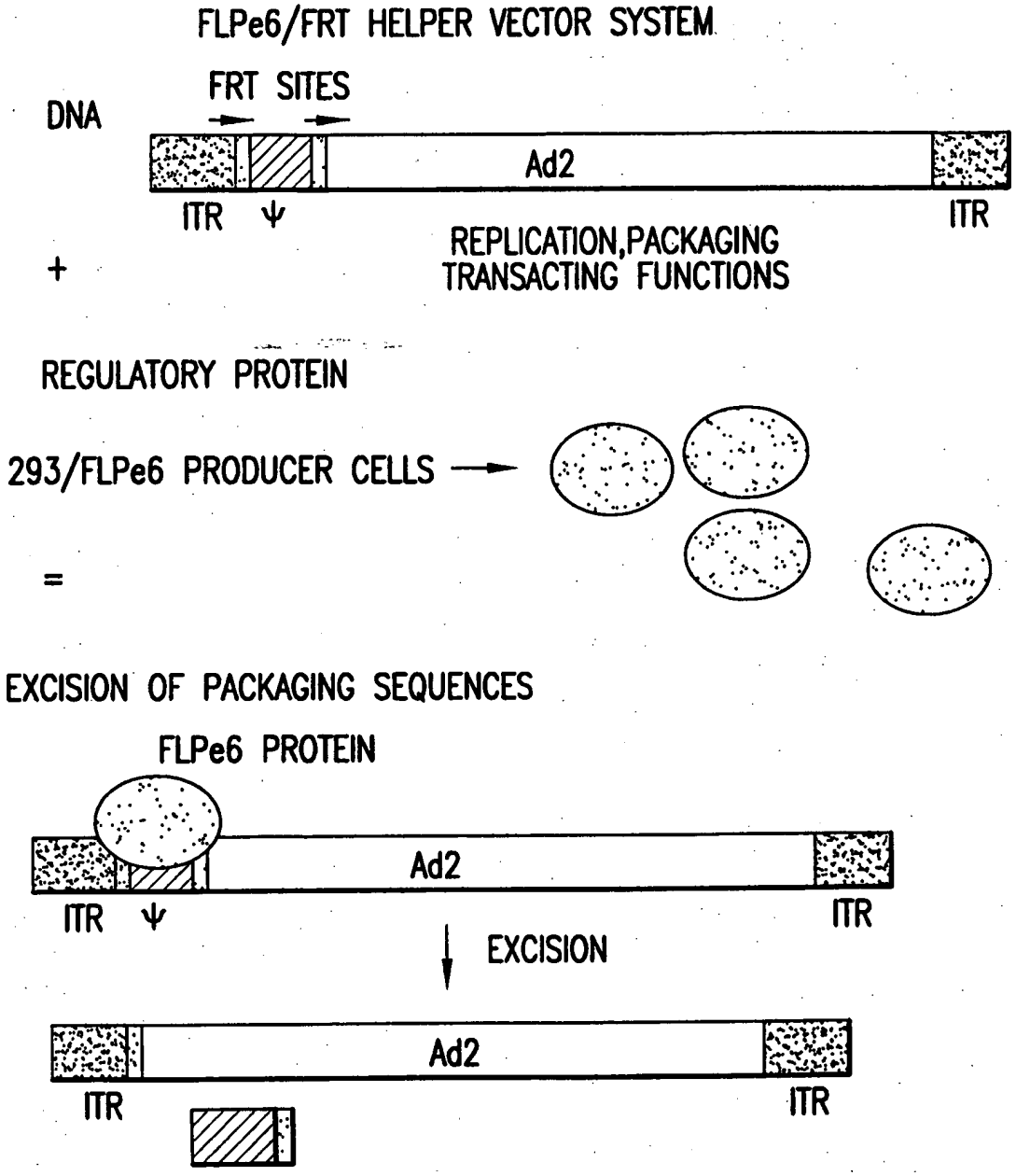
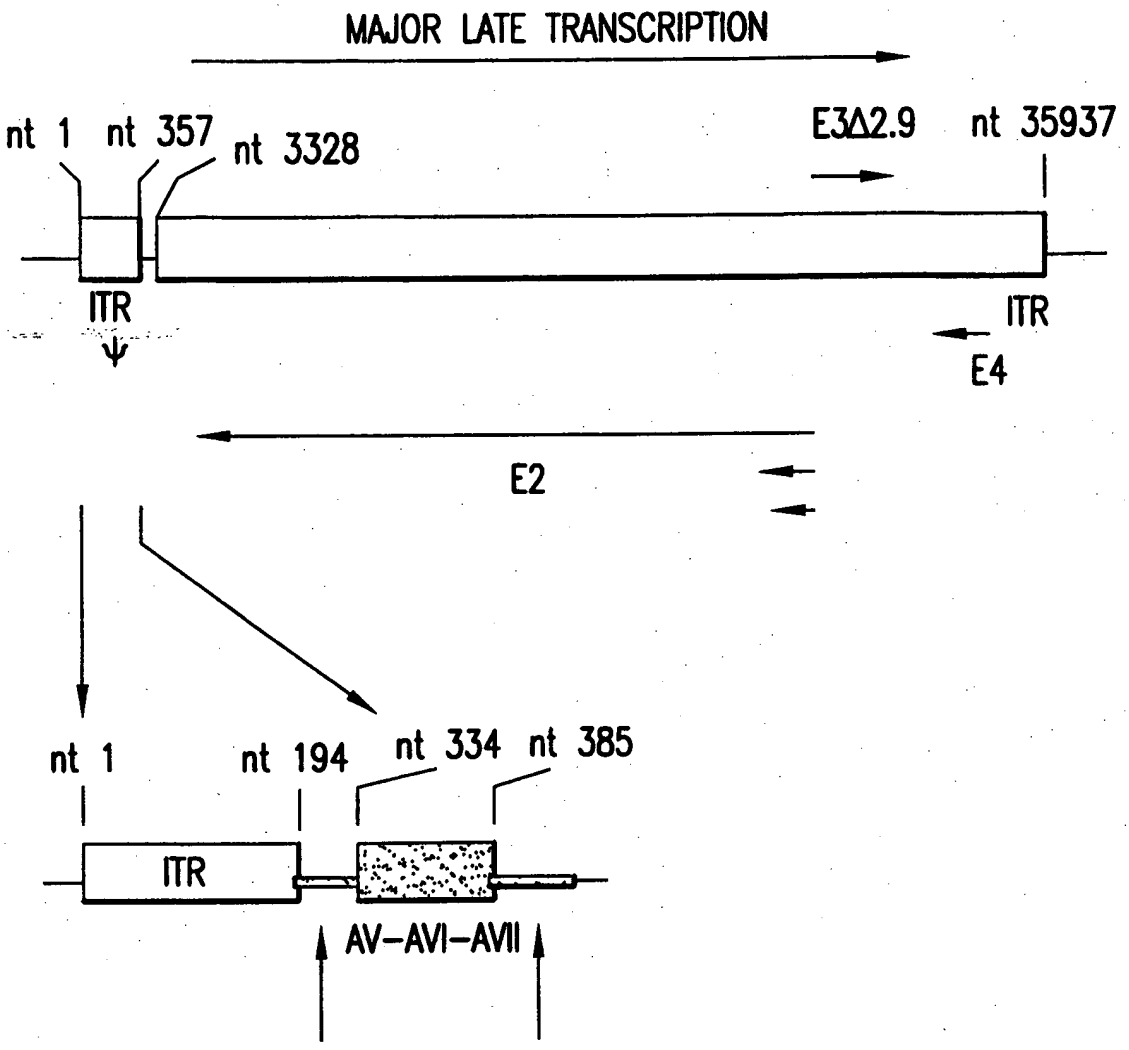

FLPe6/FRT HELPER VECTOR SYSTEM
FRT SITES
DNA
Ad2
ITR
Ψ
ITR
REPLICATION,PACKAGING
TRANSACTING FUNCTIONS
+
REGULATORY PROTEIN
293/FLPe6 PRODUCER CELLS
=
EXCISION OF PACKAGING SEQUENCES
FLPe6 PROTEIN
Ad2
ITR
Ψ
ITR
EXCISION
Ad2
ITR
ITR

FIG.2



OPERATOR SITES FOR LAMBDA REPRESSOR

HELPER VECTOR Ad(AV–AVII).

FIG.3A

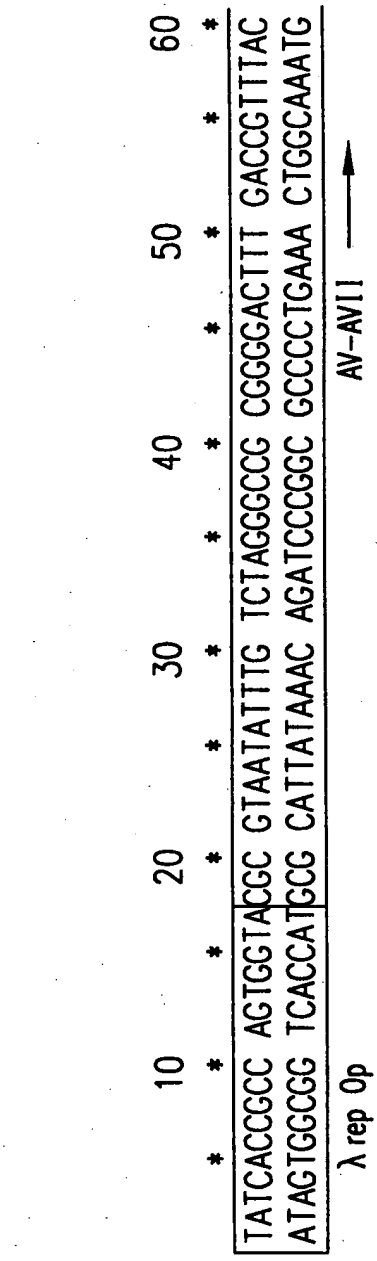
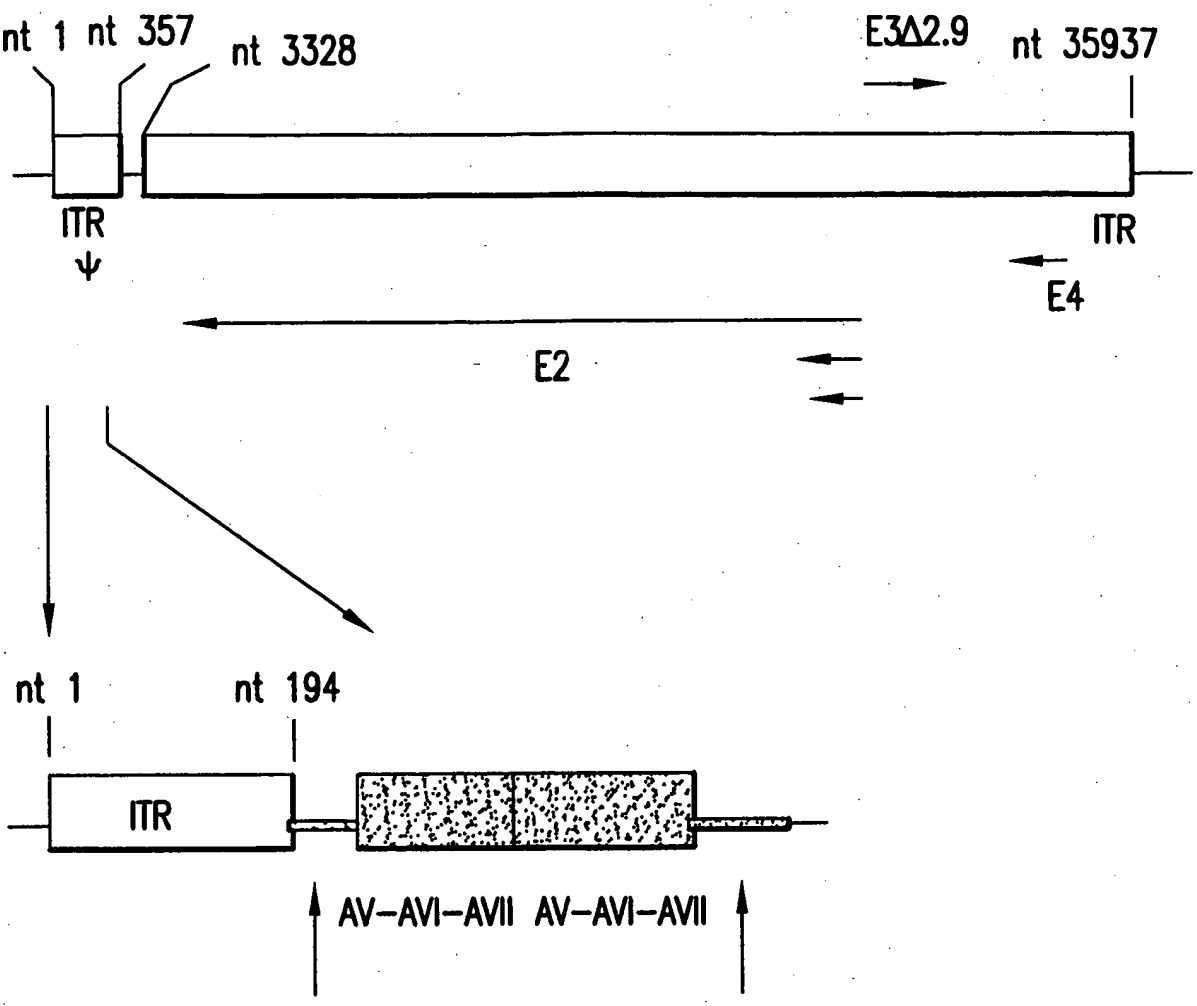

10 20 30 40 50 60
TATCACCGCC AGTGGTACGC GTAATATTTG TCTAGGGCCG CGGGGACTTT GACCGTTTAC
ATAGTGGCGG TCACCATGCG CATTATAAAC AGATCCCGGC GCCCCTGAAA CTGGCAAATG
λ rep Op
AV-AVII
70 80
GTGGAGACTT ATCACCGCCA GTGGTA
CACCTCTGAA TAGTGGCGGT CACCAT
λ rep Op

FIG.3B



OPERATOR SITES FOR LAMBDA REPRESSOR

HELPER VECTOR Ad(AV-AVII)2.

FIG.4A

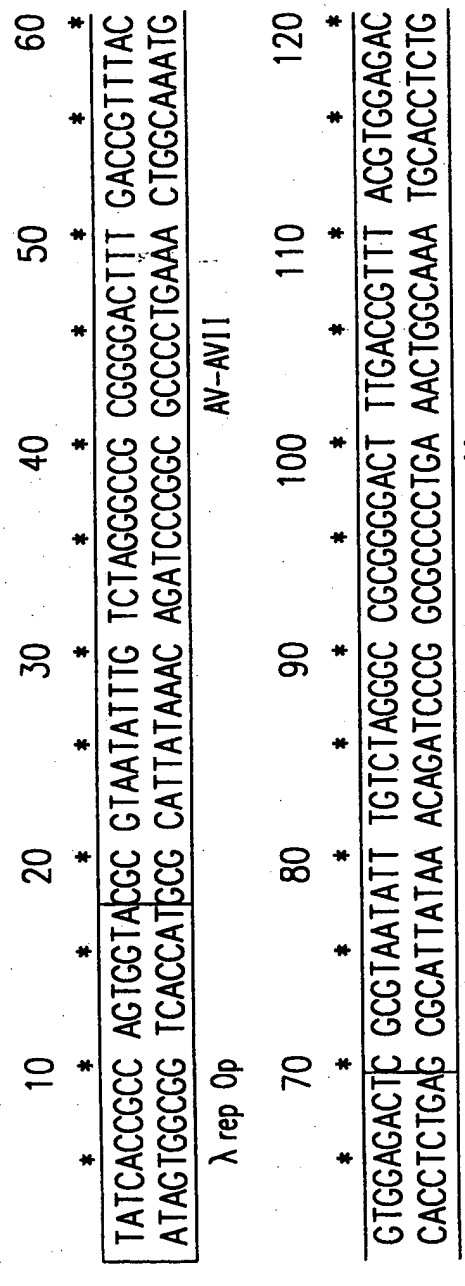

10 20 30 40 50 60
TATCACCGCC AGTGGTACGC GTAATATTTG TCTAGGGCCG CGGGGACTTT GACCGTTTAC
ATAGTGGCGG TCACCATGCG CATTATAAAC AGATCCCGGC GCCCCTGAAA CTGGCAAATG
λ rep Op
AV-AVII
70 80 90 100 110 120
GTGGAGACTC GCGTAATATT TGTCTAGGGC CGCGGGGACT TTGACCGTTT ACGTGGAGAC
CACCTCTGAG CGCATTATAA ACAGATCCCG GCGCCCCTGA AACTGGCAAA TGCACCTCTG
AV-AVII
130
TTTATCACCGC CAGTGGTA
AATAGTGGCG GTCACCAT
λ rep Op

FIG.4B

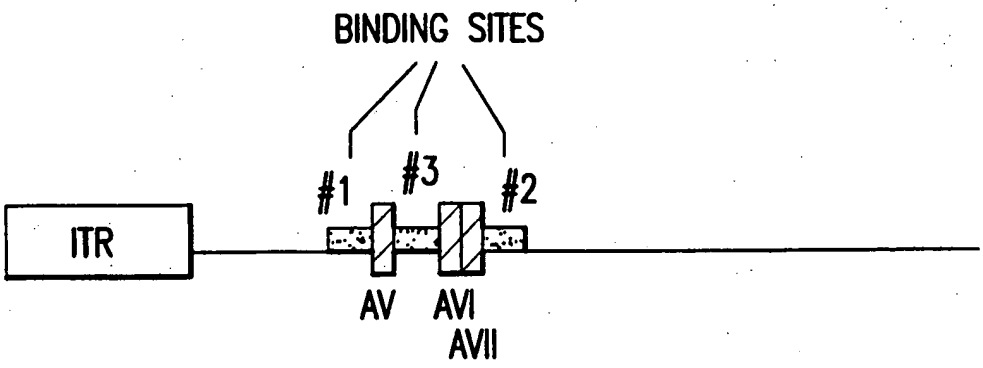

BINDING SITES
#1
#3
#2
ITR
AV
AVI
AVII

FIG.5

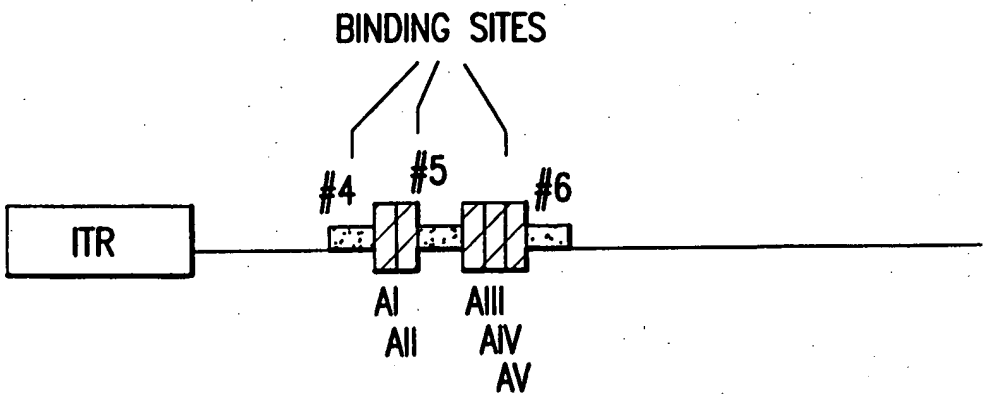

BINDING SITES
#4
#5
#6
ITR
AI
AII
AIII
AIV
AV

FIG.6

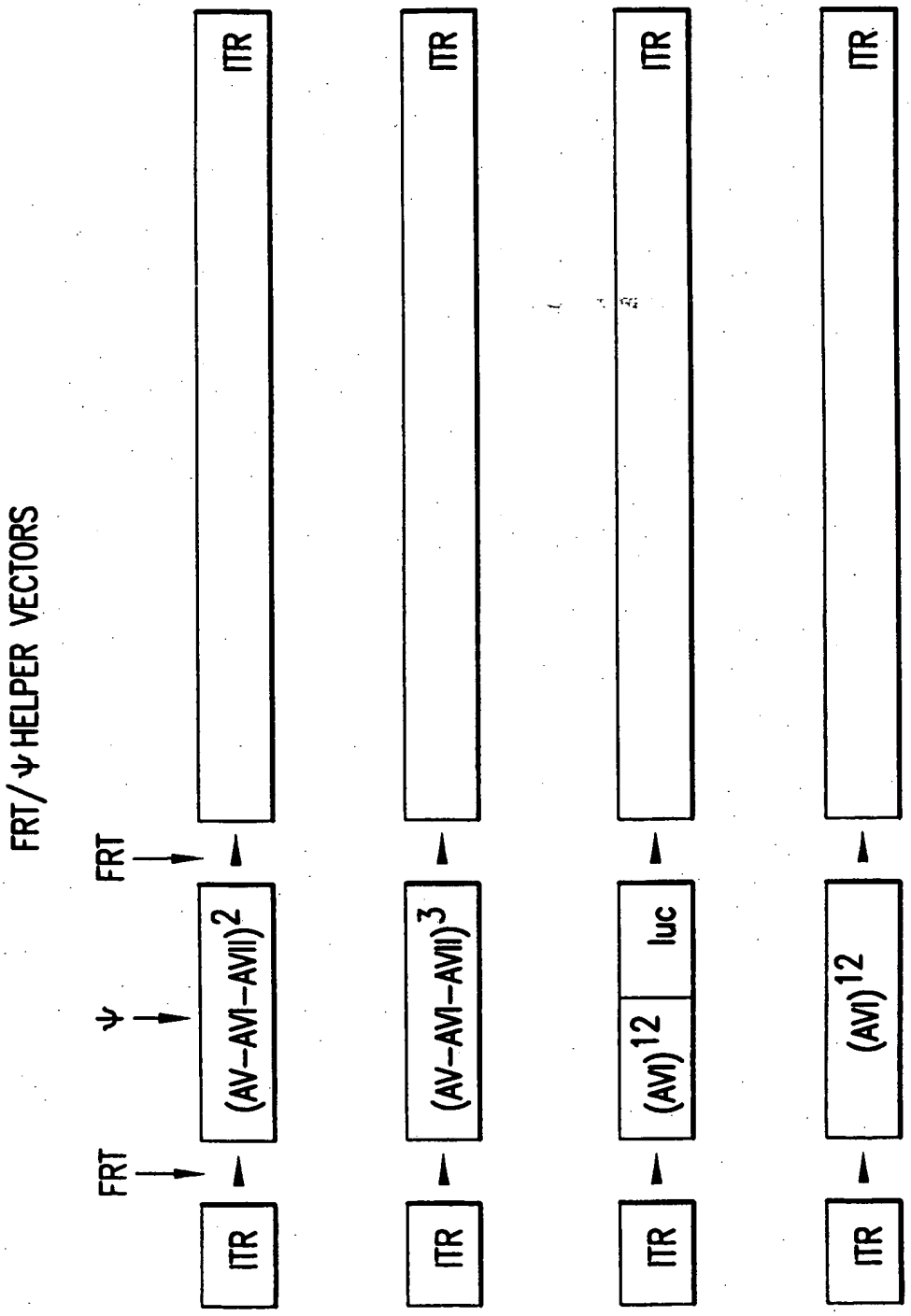

FRT/ψ HELPER VECTORS
ITR
FRT
ψ
(AV-AVI-AVII)2
FRT
ITR
ITR
(AV-AVI-AVII)3
ITR
ITR
(AVI)12
luc
ITR
ITR
(AVI)12
ITR

FIG.7

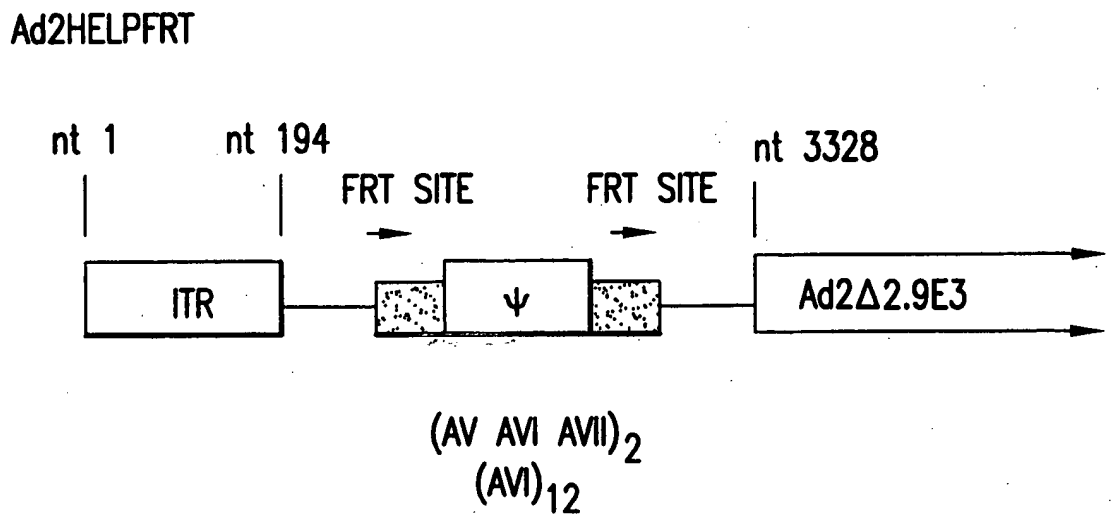

Ad2HELPFRT
nt 1
nt 194
FRT SITE
FRT SITE
nt 3328
ITR
Ψ
Ad2Δ2.9E3
(AV AVI AVII)2
(AVI)12
PACKAGING SEQUENCES
334
385
(AV–AVI–AVII)2
364
377
(AVI)12

FIG.8

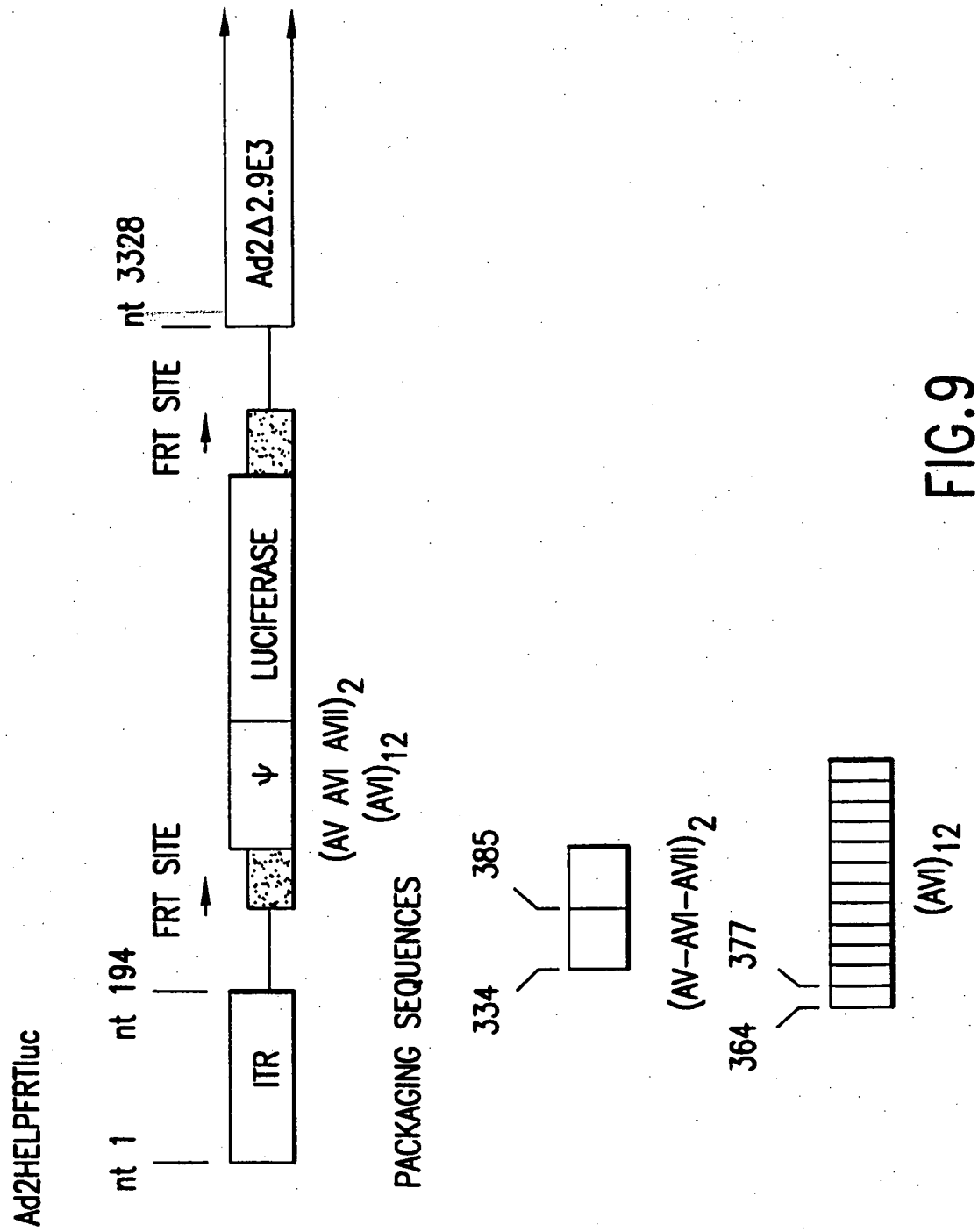

Ad2HELPFRTluc
nt 1
nt 194
FRT SITE
ITR
ψ
LUCIFERASE
FRT SITE
nt 3328
Ad2Δ2.9E3
PACKAGING SEQUENCES
(AV AVI AVII)2
(AVI)12
334
385
(AV-AVI-AVII)2
364
377
(AVI)12

FIG.9

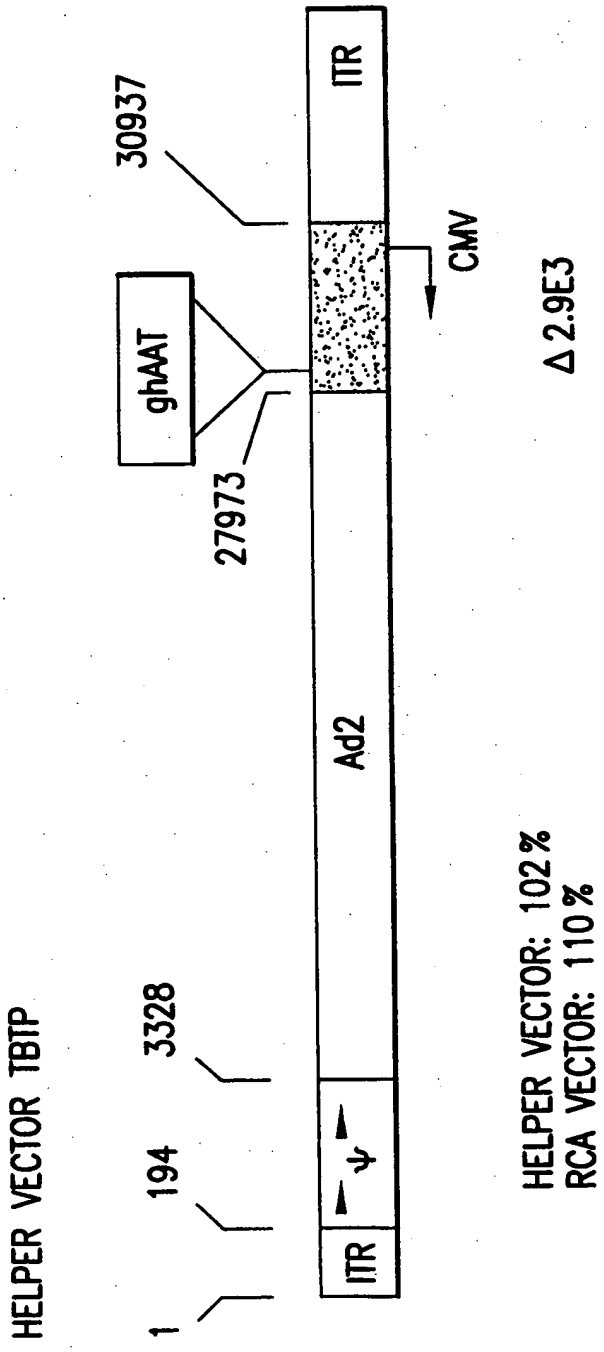

HELPER VECTOR TBTP
1
194
3328
ITR
Ψ
Ad2
27973
ghAAT
30937
ITR
CMV
Δ 2.9E3
HELPER VECTOR: 102%
RCA VECTOR: 110%

FIG.10

REPLICATION OF HELPER VECTOR WITH BINDING SITES FLANKING PACKAGING SIGNAL, PLUS AN ADDITIONAL, INTERNAL ITR_L

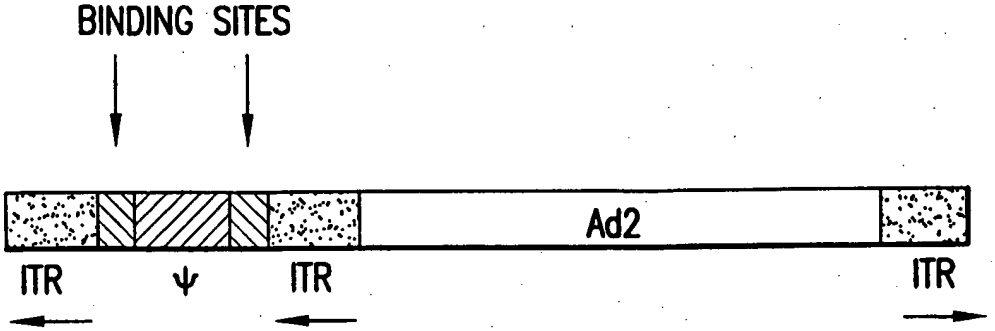


FIG.11A

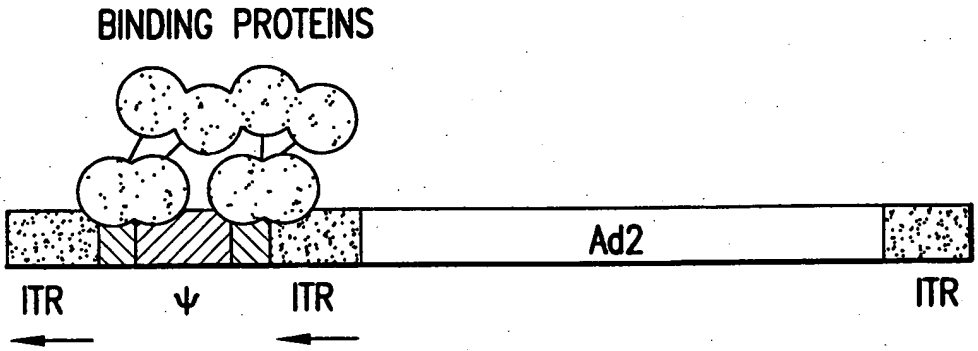
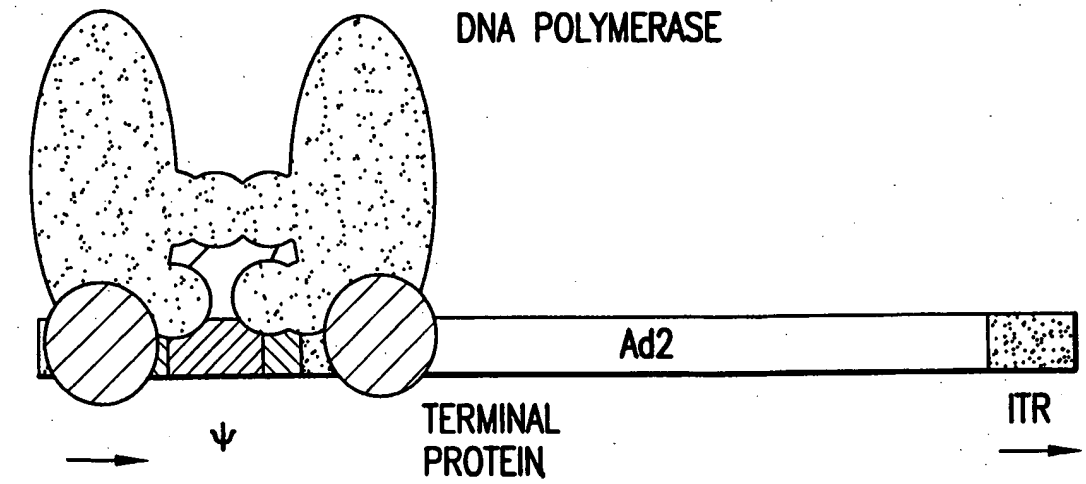


FIG.11B



REPLICATION BLOCKED

REPLICATION PROCEEDS

FIG.11C

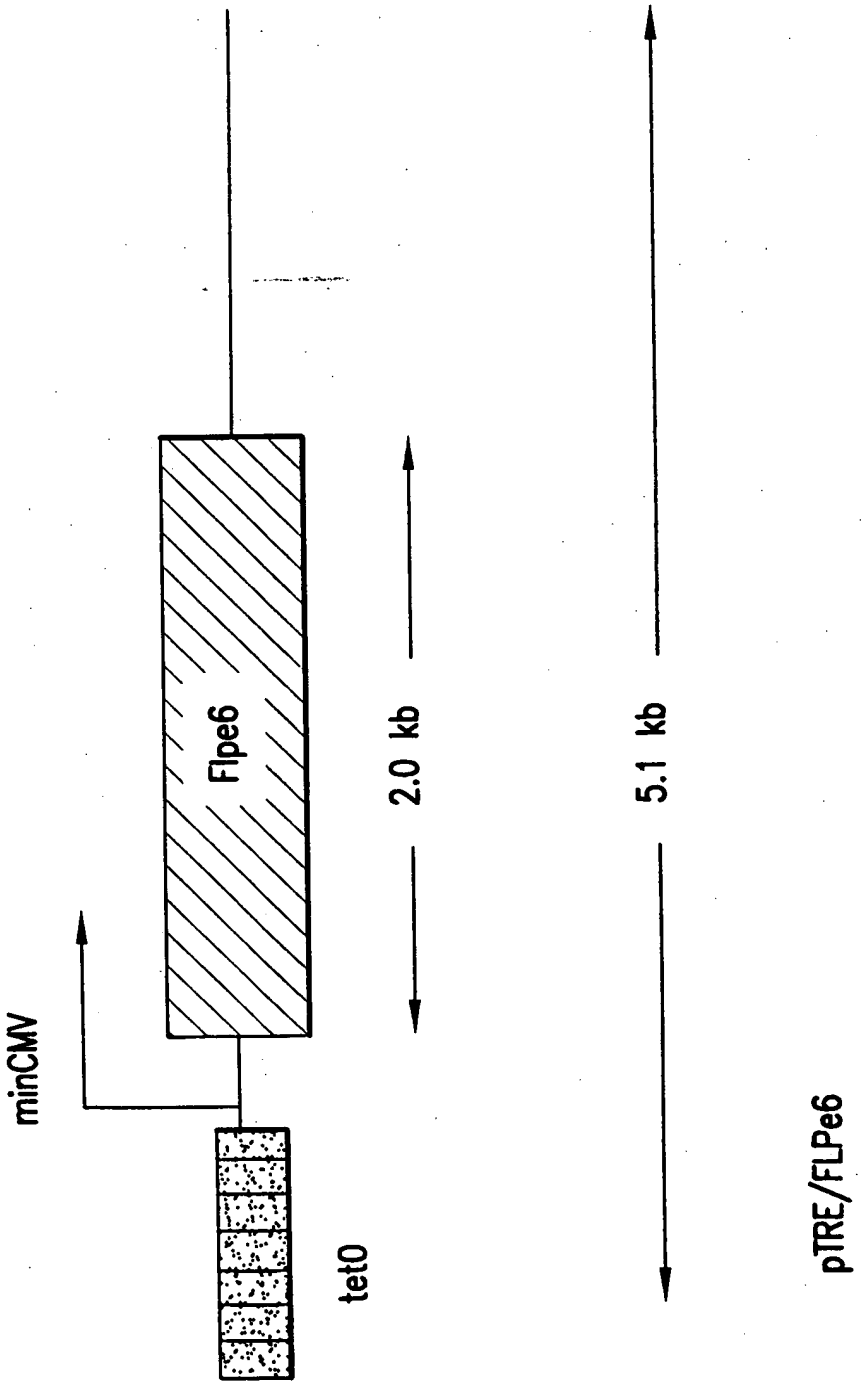


FIG.12

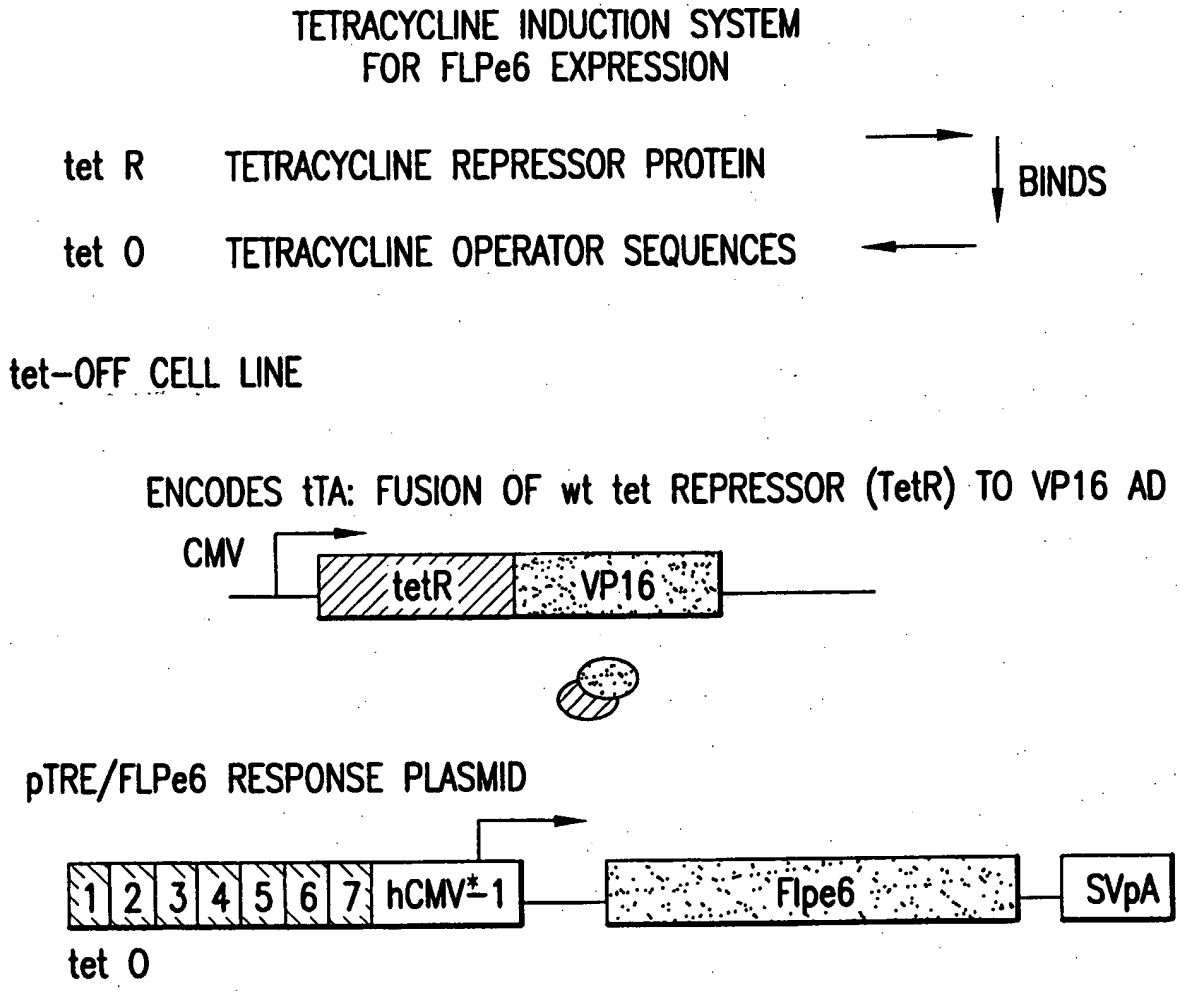

TETRACYCLINE INDUCTION SYSTEM
FOR FLPe6 EXPRESSION
tet R
TETRACYCLINE REPRESSOR PROTEIN
BINDS
tet O
TETRACYCLINE OPERATOR SEQUENCES
tet-OFF CELL LINE
ENCODES tTA: FUSION OF wt tet REPRESSOR (TetR) TO VP16 AD
CMV
tetR
VP16
pTRE/FLPe6 RESPONSE PLASMID
1 2 3 4 5 6 7
hCMV*-1
Flpe6
SVpA
tet O

FIG.13A

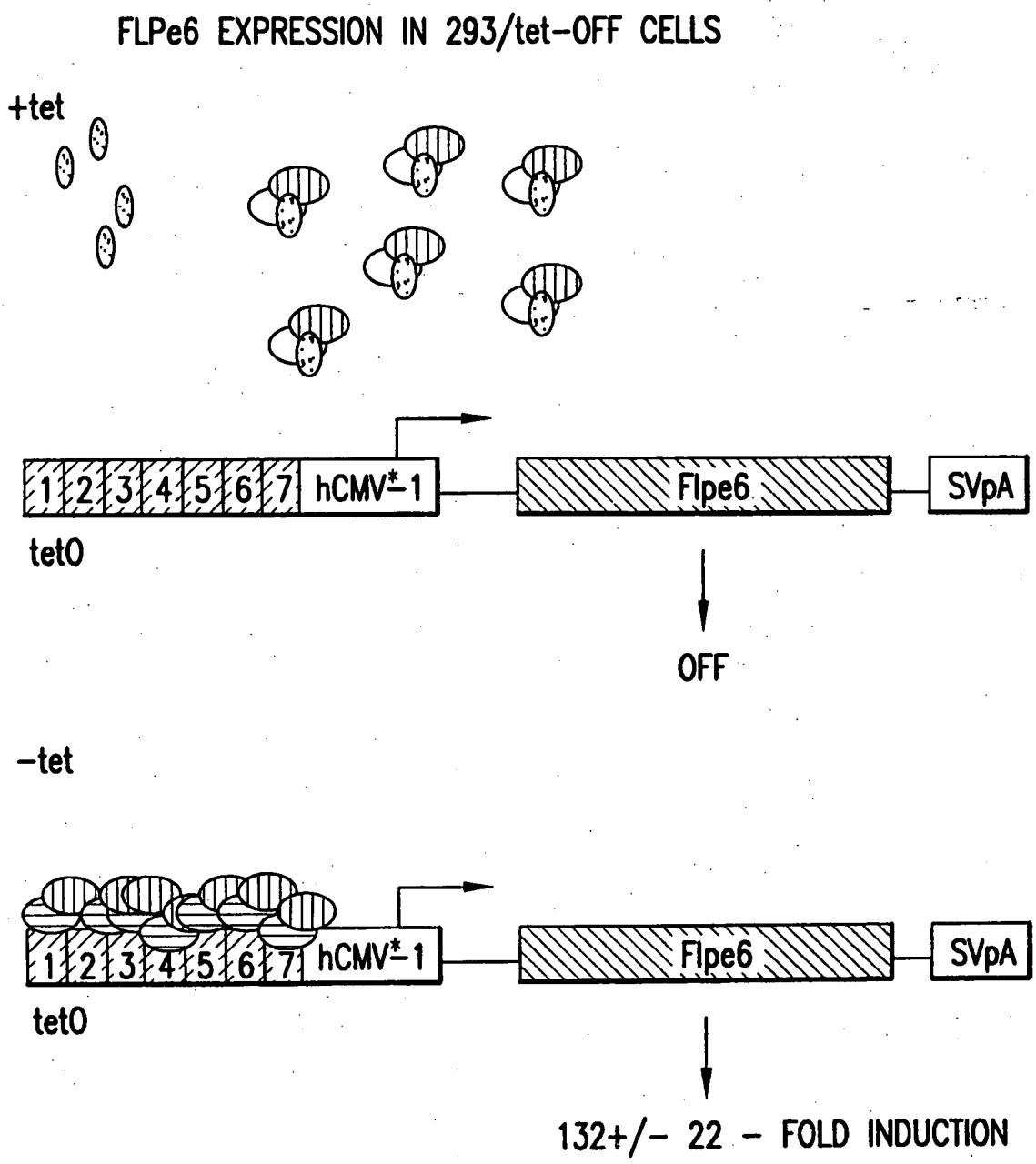
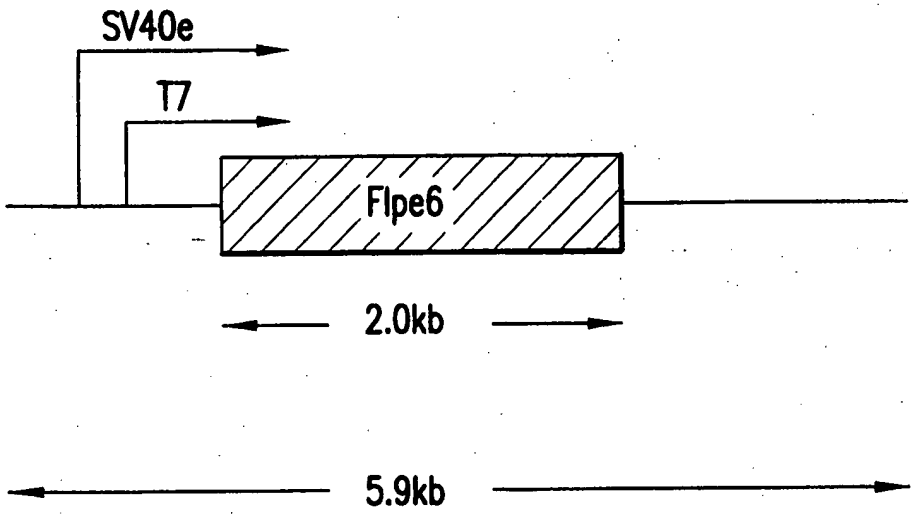

FLPe6 EXPRESSION IN 293/tet-OFF CELLS
+tet
1 2 3 4 5 6 7
hCMV*-1
Flpe6
SVpA
tetO
OFF
-tet
1 2 3 4 5 6 7
hCMV*-1
Flpe6
SVpA
tetO
132+/- 22 - FOLD INDUCTION

FIG.13B



pSVK/FLPe6

FIG.14

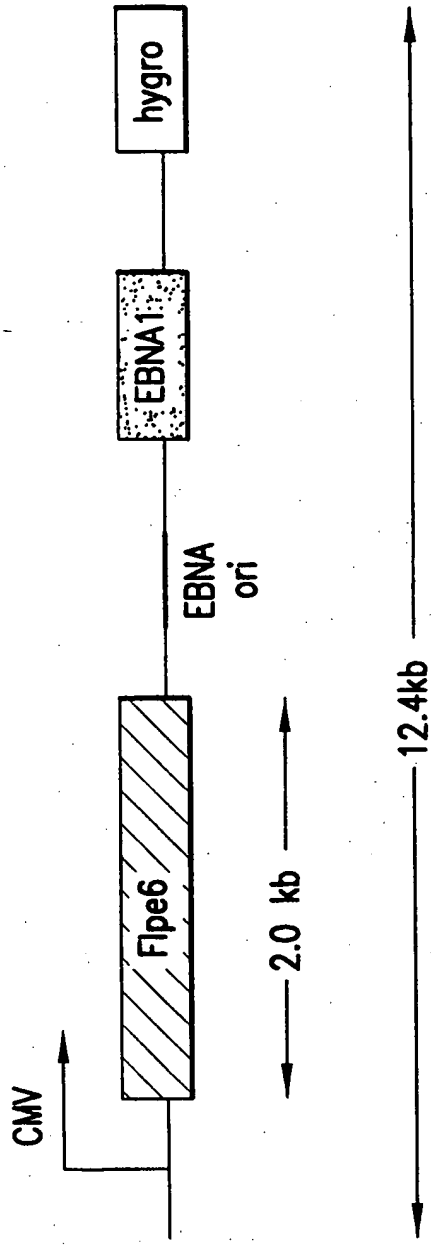


FIG.15

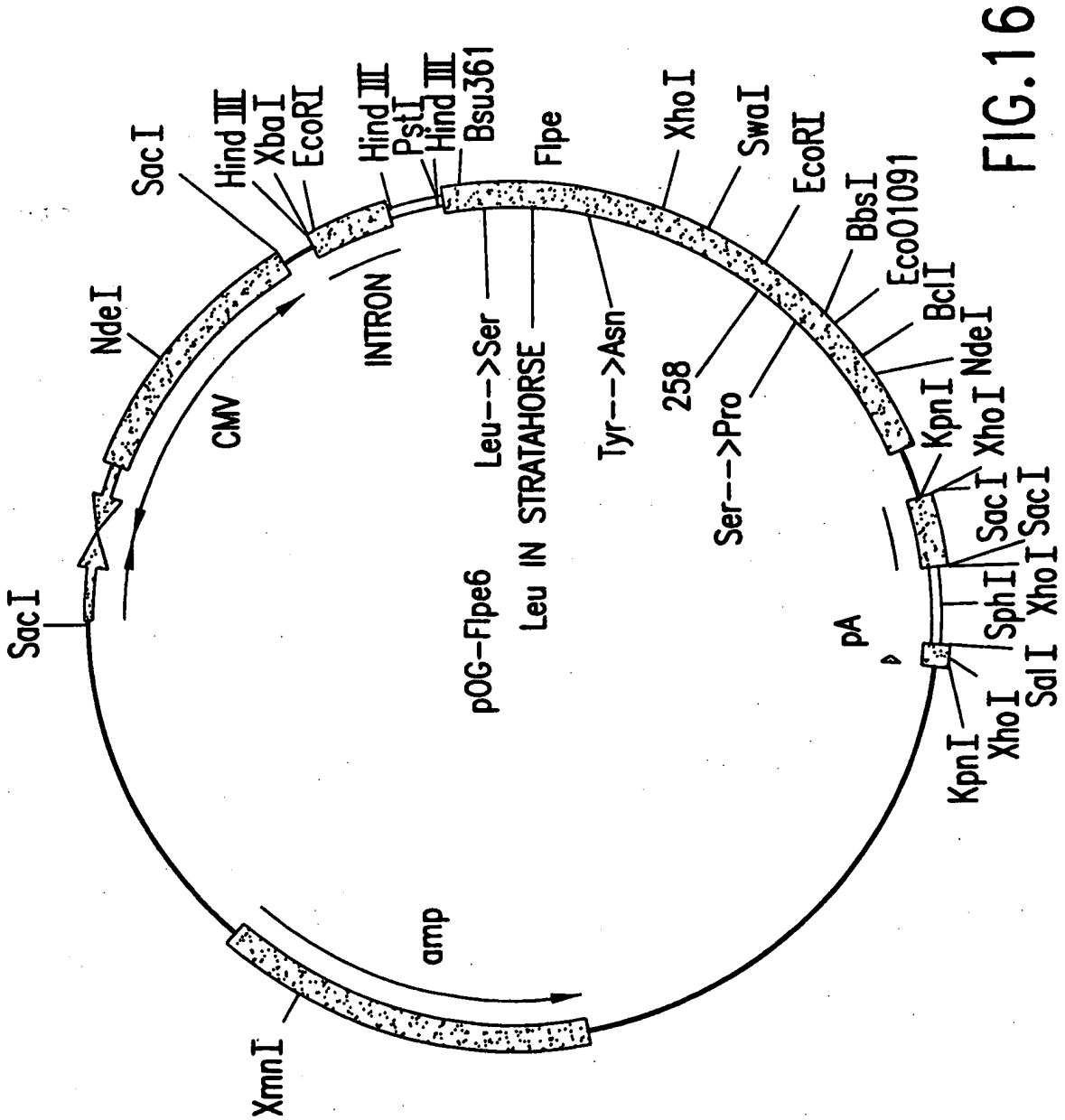

SacI
NdeI
CMV
INTRON
Leu-->Ser
Leu IN STRATAHORSE
pOG-Flpe6
Tyr-->Asn
258
Ser-->Pro
pA
amp
XmnI
SacI
HindIII
XbaI
EcoRI
HindIII
PstI
HindIII
Bsu361
Flpe
XhoI
SwaI
EcoRI
BbsI
Eco01091
BclI
NdeI
KpnI
XhoI
SacI
SacI
SphI
XhoI
SalI
KpnI

FIG.16

SEQUENCE LISTING

<110> Samuel C. Wadsworth
Helen Romanczuk
Richard J. Gregory
Donna Armentano

<120> METHODS FOR PSEUDOADENOVIRAL VECTOR PRODUCTION

<130> 31428-A-A-pct

<150> 60/074,761
<151> 1998-02-17

<150> 60/086,528
<151> 1998-05-22

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<210> 2

<211> 17

<212> DNA

<213> LAMBDA

<400> 2

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<210> 3

<211> 17

<212> DNA

<213> LAMBDA

<400> 3

tatctctggc ggtgttg 17

<210> 4

<211> 17

<212> DNA

<213> LAMBDA

<400> 4

tatcaccgca gatggtt 17

<210> 5

<211> 17

<212> DNA

<213> LAMBDA

<400> 5

taacaccgtg cgtgttg 17

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<212> DNA

<213> PAPILLOMA VIRUS

<400> 7

accgaaatcg gt 12

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<400> 8

accgaaaccg gt 12

<210> 9

<211> 12

<212> DNA

<213> papilloma virus

<400> 9

accnnnnnng gt 12

<210> 10

<211> 48

<212> DNA

<213> yeast

<400> 10

gaagttccta ttccgaagtt cctattctct agaaagtata ggaacttc 48

<210> 11

<211> 35

<212> DNA

<213> yeast

<400> 11

cgaagttcct attctctaga aagtatagga acttc 35

<210> 12

<211> 54

<212> DNA

<213> Artificial Sequence

<220>

<223> synthesized as a hybrid of yeast and adenovirus sequences

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gtataggaac ttca 54

<210> 13

<211> 54

<212> DNA

<213> Artificial Sequence

<220>

<223> synthesized as a hybrid of yeast and adenovirus sequences

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gaataggaac ttca 54

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aagtatagga acttca 96

<210> 15

<211> 96

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gacaaatatt acgcga 96

<210> 16

<211> 21

<212> DNA

<213> adenovirus

<400> 16

tcgaccgcgg ggactttgac c 21

<210> 17

<211> 21

<212> DNA

<213> adenovirus

<400> 17

tcgaggtcaa agtccccgcg g 21

<210> 18

<211> 54

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adenovirus sequences

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gtataggaac ttcc 54

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gaataggaac ttca 54

<210> 20

<211> 54

<212> DNA

<213> Artificial Sequence

<220>

<223> synthesized as a hybrid of yeast and adenovirus sequences

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gtataggaac ttca 54

<210> 21

<211> 54

<212> DNA

<213> Artificial Sequence

<220>

<223> synthesized as a hybrid of yeast and adenovirus sequences

<400> 21

cgcgtgaagt tcctatactt tctagagaat aggaacttcg 40

gaataggaac ttcc 54

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/03483

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR 2 737 501 A (TRANSGENE SA) 7 February 1997 see page 5, line 9 - line 17 see page 8, line 17 - line 20 see page 9, line 18 - page 10, line 11 ---	1,4,5, 10,15, 17-25
X	WO 97 32481 A (UNIV CALIFORNIA ;HARDY STEPHEN F (US)) 12 September 1997 see the whole document, especially page 9, lines 1-3 --- -/--	1,4,5, 10,15, 17-19, 22,23 20,21, 24,25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 July 1999	20/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mandl, B

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/03483

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	GOSSEN M. ET AL.: "TRANSCRIPTIONAL ACTIVATION BY TETRACYCLINES IN MAMMALIAN CELLS" SCIENCE, vol. 268, 23 June 1995, pages 1766-1769, XP002014056 see the whole document	20,21, 24,25
X	PARKS R. J. ET AL.: "A HELPER-DEPENDENT ADENOVIRUS VECTOR SYSTEM: REMOVAL OF HELPER VIRUS BY CRE-MEDIATED EXCITION OF THE VIRAL PACKAGING SIGNAL" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, no. 24, 26 November 1996, pages 13565-13570, XP000617948 cited in the application see the whole document	15
X	HARDY S ET AL: "CONSTRUCTION OF ADENOVIRUS VECTORS THROUGH CRE-LOX RECOMBINATION" JOURNAL OF VIROLOGY, vol. 71, no. 3, 1 March 1997, pages 1842-1849, XP000670223 cited in the application see the whole document	15
A	SCHMID S. I. AND HEARING P.: "Bipartite structure and functional independence of adenovirus 5 packaging elements." JOURNAL OF VIROLOGY, vol. 71, no. 5, May 1997, pages 3375-3384, XP002108134 cited in the application see abstract see page 3379, left-hand column, last paragraph - right-hand column, paragraph 3	5,6
A	JOHNSON A. D. ET AL.: "LAMBDA REPRESSOR AND CRO-COMPONENTS OF AN EFFICIENT MOLECULAR SWITCH" NATURE, vol. 294, no. 5838, 1 November 1981, pages 217-223, XP002049066 see page 217, left-hand column, last paragraph - page 218, right-hand column, paragraph 3	2,3,9, 11,13,16

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